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Rev. 10/97
Modified PTO 1083

12/18/01

PATENTS

Attorney Docket No. STK-001CP2DVEWC PTE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Patent No.	:	5,258,494
Issued	:	November 2, 1993
Inventors	:	Hermann Oppermann et al.
Assignee	:	Stryker Corporation
Filed	:	December 22, 1992
Title	:	OSTEOGENIC PROTEINS

Hon. Commissioner for Patents
Washington, D.C. 20231

New York, New York
December 14, 2001

Attn: BOX PATENT EXTENSION

EXPRESS MAIL CERTIFICATION

"Express Mail" mailing label number EI125454393US.

Date of Deposit December 14, 2001 .

I hereby certify that this transmittal letter and the other papers and fees identified in this transmittal letter as being transmitted herewith are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10 on the date indicated above and are addressed to the Commissioner for Patents, Washington, D.C. 20231.

Lillian Garcia
Lillian Garcia

TRANSMITTAL LETTER

Sir:

Transmitted herewith is an Application for Extension of Patent Term Pursuant to 35 U.S.C. § 1.56(d) and 37 C.F.R. § 1.740 (including Appendices A-Y) for the above-identified patent. Also enclosed herewith pursuant to 37 C.F.R. § 1.740(b) are two additional copies of the Application for a total of three copies. This Application

is being submitted within the 60 day period permitted for submission pursuant to 37 C.F.R. § 1.720(f). The last day upon which this application could be submitted is December 16, 2001 (a Sunday).

In accordance with 37 C.F.R. § 1.740(a)(14), a check in the amount of $1,120.00 in payment of the fee pursuant to 37 C.F.R. § 1.20(j) is enclosed herewith. The Director is authorized to charge any additional fee to Deposit Account No. 06-1075. A duplicate copy of this transmittal letter is transmitted herewith.

Respectfully submitted,

Karen Mangasarian

James F. Haley, Jr. (Reg. No. 27,794)
Karen Mangasarian (Reg. No. 43,772)
Atttorney for Applicants
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Rev. 10/97
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Respectfully submitted,

James F. Haley, Jr. (Reg. No. 27,794)
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STK-001CP2DVFWC PTE

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Patent No.	:	5,258,494
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Inventors	:	Hermann Oppermann et al.
Assignee	:	Stryker Corporation
Filed	:	December 22, 1992
Title	:	OSTEOGENIC PROTEINS

New York, New York
December 14, 2001

Hon. Commissioner for Patents
Washington, D.C. 20231
Box Patent Extension

APPLICATION FOR EXTENSION OF PATENT TERM
PURSUANT TO 35 U.S.C. § 1.56(d)(1) AND 37 C.F.R. § 1.740

Sir:

Applicant, Stryker Corporation, represents that it is the assignee of the entire interest in and to the above-identified patent, recorded May 17, 1991 at reel 5863, frame 0091. Applicant hereby requests that the term of the above-identified United States patent be extended pursuant to 35 U.S.C. § 156. The information as required by 37 C.F.R. § 1.740 is provided below and in the attached Appendices.

(1) COMPLETE IDENTIFICATION OF THE APPROVED PRODUCT

The approved product OP-1™ IMPLANT ("Product") comprises recombinant human osteogenic protein-1 (OP-1)(also referred to as bone morphogenic protein-7 (BMP-7)) and bovine bone derived collagen. The amino acid sequence of human OP-1 is provided in Appendix A. Characteristics of

the Product are provided in the package insert for OP-1 Implant (Appendix B).

(2) COMPLETE IDENTIFICATION OF THE FEDERAL STATUTE UNDER WHICH THE REGULATORY REVIEW OCCURRED

The regulatory review of the Product occurred under Section 515 and 520(m) of the Federal Food, Drug, and Cosmetic Act.

(3) IDENTIFICATION OF THE DATE ON WHICH THE PRODUCT RECEIVED PERMISSION FOR COMMERCIAL MARKETING OR USE

The date on which the Product received permission for commercial marketing is October 17, 2001 (see Appendix C).

(4) IDENTIFICATION OF EACH ACTIVE INGREDIENT IN THE PRODUCT AND AS TO EACH ACTIVE INGREDIENT, A STATEMENT THAT IT HAS NOT BEEN PREVIOUSLY APPROVED FOR COMMERCIAL MARKETING OR USE

The active ingredient in the Product is osteogenic protein-1 (OP-1) (also referred to as bone morphogenic protein-7 (BMP-7)). OP-1 has not been previously approved for commercial marketing or use under the Federal Food, Drug, and Cosmetic Act, the Public Health Service Act, or the Virus-Serum-Toxin Act.

(5) STATEMENT THAT THIS APPLICATION FOR PATENT TERM EXTENSION IS BEING SUBMITTED WITHIN THE SIXTY DAY PERIOD AND IDENTIFICATION OF THE LAST DAY ON WHICH THE APPLICATION COULD BE SUBMITTED

This application is being submitted within the 60 day period permitted for submission pursuant to 37 C.F.R. § 1.720(f). The last date upon which this application could be submitted is December 16, 2001 (a Sunday).

(6) COMPLETE IDENTIFICATION OF THE PATENT FOR WHICH AN EXTENSION IS BEING SOUGHT

The United States patent for which an extension is being sought is in the names of the inventors Hermann Oppermann, Thangavel Kuberasampath, David C. Rueger and Engin Ozkaynak. The. It issued on November 2, 1993 as U.S. patent 5,258,494 ("the Patent") and will expire on November 2, 2010.

(7) COPY OF THE PATENT FOR WHICH AN EXTENSION IS BEING SOUGHT

A complete copy of the Patent is submitted herewith as Appendix D.

(8) COPY OF ANY DISCLAIMER, CERTIFICATE OF CORRECTION, RECEIPT OR MAINTENANCE FEE PAYMENT OR REEXAMINATION CERTIFICATE ISSUED IN THE PATENT

No certificate of correction was filed for the Patent.

No reexamination was filed for the Patent.

A copy of a receipt for a maintenance fee paid on May 1, 1997 is provided herewith as Exhibit E.

A copy of a receipt for a maintenance fee paid on May 1, 2001 is provided herewith as Exhibit F.

No disclaimer has been made for the Patent.

(9) STATEMENT THAT THE PATENT CLAIMS THE APPROVED PRODUCT OR A METHOD OF USING OR MANUFACTURING THE APPROVED PRODUCT, AND A SHOWING WHICH LISTS EACH APPLICABLE PATENT CLAIM AND DEMONSTRATES THE MANNER IN WHICH EACH APPLICABLE PATENT CLAIM READS ON THE APPROVED PRODUCT OR METHOD OF USING OR MANUFACTURING THE APPROVED PRODUCT

The approved product is covered by claims 1-4, 6-9, 11-12 and 16 of the Patent.

CLAIM 1	**Elements of the Product**
A protein produced by expression of recombinant DNA in a host cell and isolated from said host cell, comprising	OP-1 of the Product is recombinantly produced and isolated from CHO cells (see Appendix A).
a pair of polypeptide chains disulfide bonded to produce a dimeric species	OP-1 of the Product is a dimeric protein comprising a pair of disulfide bonded polypeptide chains (see Appendix A).
each of said pair of polypeptide chains having less than 200 amino acids	OP-1 of the Product consists of two 139 amino acid polypeptide chains each of which consists of amino acid residues 1-139 in Figure 1.B.5 in Appendix A.
in a sequence sufficiently duplicative of COP-5.... or COP-7....,	OP-1 of the Product is sufficiently duplicative of COP5 and COP7 (see Appendix G)
such that the dimeric species has a conformation capable of inducing endochondral bone and cartilage formation when implanted in a mammal in association with a matrix.	Dimeric OP-1 of the Product is capable of inducing endochondral bone and cartilage formation (see Appendix H and I).

CLAIM 2	Elements of the Product
The osteogenic protein of claim 1	See claim 1 above.
having a molecular weight of about 30kD when oxidized as determined by comparison to molecular weight standards in SDS-polyacrylamide gel.	OP-1 of the Product has a molecular weight of approximately 30kD as determined by SDS-polyacrylamide gel electrophoresis (see Appendix J).

CLAIM 3	Elements of the Product
The osteogenic protein of claim 2	See claim 2 above.
further characterized by being glycosylated.	OP-1 of the Product is glycosylated (see Appendix K).

CLAIM 4	Elements of the Product
The osteogenic protein of claim 1	See claim 1 above.
having a molecular weight of about 27 kD as determined by comparison to molecular weight standards in SDS-polyacrylamide gel electrophoresis.	OP-1 of the Product has a molecular weight of approximately 27 kD as determined by SDS-polyacrylamide gel electrophoresis (see Appendix J).

CLAIM 6	Elements of the Product
The protein of claim 1 comprising	See claim 1 above.
the amino acid sequences: CXXXXLXVXFXDXGWXXWXXXPXGXXAX YCXGXCXXPXXXXXXXXNHAXXQXXVXX XNXXXXPXXCCXPXXXXXXXXLXXXXXX XVXLXXYXXMXVXXCXCX wherein each X independently represents an amino acid.	OP-1 comprises the sequence **C**KKH**E**L**Y**V**S**F**R**D**L**G**W**QD**W**II**A**P**E**GY**A**A**Y** **YC**E**GEC**AF**P**LNSYMNAT**NH**AIV**Q**TLVHF **I**N**P**ETV**P**KP**CCA**PTQLNAISV**L**YFDDSS **N**V**IL**KK**Y**RN**M**V**V**RA**C**G**C**H (see Appendix A).

CLAIM 7	**Elements of the Product**
The protein of claim 1 comprising	See claim 1 above.
the amino acid sequences: LXVXFXDXGWXXWXXXPXGXXAXYCXGX CXXPXXXXXXXXNHAXXQXXVXXXNXXX XPXXCCXPXXXXXXXXLXXXXXXXVXLX XYXXMXVXXCXCX wherein each X independently represents an amino acid.	OP-1 of the Product comprises the sequence **LYVSFRDLGWQDWIIAPEGYAAYYCEGE CAFPLNSYMNATNHAIVQTLVHFINPET VPKPCCAPTQLNAISVLYFDDSSNVILK KYRNMVVRACGCH** (see Appendix A).

CLAIM 8	**Elements of the Product**
The protein of claim 1 comprising	See claim 1 above.
the amino acid sequence: LYVSFRDLGWQDWIIAPEGYAAYYCEGE CAFPLNSYMNATNHAIVQTLVHFINPET VPKPCCAPTQLNAISVLYFDDSSNVILK KYRNMVVRACGCH.	OP-1 of the Product comprises the sequence LYVSFRDLGWQDWIIAPEGYAAYYCEGE CAFPLNSYMNATNHAIVQTLVHFINPET VPKPCCAPTQLNAISVLYFDDSSNVILK KYRNMVVRACGCH (see Appendix A).

CLAIM 11	**Elements of the Product**
The protein of claim 1	See claim 1 above.
produced by expression in a mammalian cell line.	OP-1 of the Product is expressed in CHO cells which is a mammalian cell line (see Appendix A).

CLAIM 12	**Elements of the Product**
The protein of claim 1	See claim 1 above.
having a half-maximum bone forming activity of at least 20-25 ng per 25 mg of implant.	OP-1 of the Product has a half-maximum bone forming activity of 50-100ng/25 mg of matrix (see Appendix L, abstract).

CLAIM 16	Elements of the Product
An isolated protein comprising	OP-1 of the Product is an isolated protein (see Appendix A).
a pair of polypeptide chains disulfide bonded to form a dimeric species	OP-1 is a dimeric protein comprising two polypeptide chains which are disulfide bonded (see Appendix A).
at least one of said polypeptide chains comprising the amino acid sequence: LYVSFRDLGWQDWIIAPEGYAAYYCEGE CAFPLNSYMNATNHAIVQTLVHFINPET VPKPCCAPTQLNAISVLYFDDSSNVILK KYRNMVVRACGCH.	Each of the polypeptide chains in OP-1 of the Product comprises the amino acid sequence LYVSFRDLGWQDWIIAPEGYAAYYCEGE CAFPLNSYMNATNHAIVQTLVHFINPET VPKPCCAPTQLNAISVLYFDDSSNVILK KYRNMVVRACGCH (see Appendix A).

(10) STATEMENT OF THE RELEVANT DATES AND INFORMATION PURSUANT TO 35 U.S.C. § 156(g) IN ORDER TO ENABLE THE SECRETARY OF HEALTH AND HUMAN SERVICES OR THE SECRETARY OF AGRICULTURE TO DETERMINE THE APPLICABLE REGULATORY REVIEW PERIOD

The relevant dates and information pursuant to 35 U.S.C. § 156(g) in order to enable the Secretary of Health and Human Services or the Secretary of Agriculture to determine the applicable regulatory review period are as follows:

The effective date of the investigational device exemption (IDE) was November 14, 1991. The IDE Number is G910130/A2.

The date on which the application for product approval under Section 515 of the Federal Food, Drug, and Cosmetic Act was initially submitted was April 4, 1998. The Modular PMA Submission number is M980003.

The date of the non-approval decision was January 29, 2001.

The date on which a Request for Humanitarian Use Device Designation was filed was February 20, 2001.

The date on which the HDE application was filed was May 25, 2001. The HDE number is H010002/A001.

The application was approved on October 17, 2001 under the Humanitarian Device Exemption (HDE).

(11) BRIEF DESCRIPTION OF THE SIGNIFICANT ACTIVITIES UNDERTAKEN BY THE MARKETING APPLICANT DURING THE APPLICABLE REGULATORY REVIEW PERIOD WITH RESPECT TO THE APPROVED PRODUCT AND THE SIGNIFICANT DATES APPLICABLE TO SUCH ACTIVITY

Applicant filed an investigational device exemption (IDE) on August 1, 1991 (See Appendix M).

Applicant obtained the IDE on November 14, 1991. The IDE Number was G910130/A2 (see Appendix N).

Applicant enrolled the first patient in a clinical study on February 14, 1992 (see Appendix O).

Applicant monitored the status of the patients enrolled in the clinical studies at least once a year. The clinical studies lasted until 1998 (see Appendix P).

Applicant filed the first PMA modular submission on April 4, 1998. The Modular PMA Submission number was M980003. Amendments to the submission were made on April 24, June 16 and 17 and July 31, 1998 (see Appendix Q).

Applicant filed the second PMA modular submission on May 21, 1998 (see Appendix R).

Applicant obtained acceptance of the Nonclinical Laboratory Studies Module on August 21, 1998 (see Appendix S).

Applicant filed the third PMA modular submission on June 4, 1999. The PMA number was P990029. Amendments to the submission were made on June 6, 11, and 17, August 16, and September 3, 16, and 22 (2 amendments), November 1, and December 3, 1999; March 13, June 1, 14, and 16 (3 amendments), August 8, and 16, September 6 and 12, October 6 and November 22, 2000 (see Appendix T).

Applicant obtained a decision that the PMA was not approvable on January 29, 2001 (see Appendix U).

Applicant filed a request for Humanitarian Use Device Designation on February 20, 2001 (see Appendix V).

Applicant obtained approval of the request for Humanitarian Use Device Designation on May 4, 2001 (see Appendix W).

Applicant filed an application for a Humanitarian Device Exemption (HDE) on May 25, 2001. The HDE number was H010002/A001. The application was amended on May 29, June 28, July 5 and 23, and October 9, 11 and 17, 2001 (see Appendix X).

Applicant obtained approval of the HDE application on October 17, 2001 (see Appendix Y).

(12) STATEMENT THAT IN THE OPINION OF APPLICANT THE PATENT IS ELIGIBLE FOR THE EXTENSION AND A STATEMENT AS TO THE LENGTH OF EXTENSION CLAIMED, INCLUDING HOW THE LENGTH OF EXTENSION WAS DETERMINED

In the opinion of the applicant, the Patent is eligible for patent term extension pursuant to 35 U.S.C. § 156(a) for the following reasons:

a) The term of the Patent has not expired before this application is submitted.

b) The term of the Patent has never been extended.

c) The application for patent term extension is submitted by an authorized agent of the record owner of the Patent.

d) The Product has been subject to a regulatory review period before its commercial marketing or use as evident from paragraph 11 above.

e) The approval for the commercial marketing or use of the Product after said regulatory period is the first commercial marketing or use of the Product under the provisions of the Federal Food, Drug, and Cosmetic Act.

Applicant believes that the Patent is eligible for the maximum statutory extension of 14 years from FDA approval (i.e., until October 17, 2015). This determination was made by:

(a) Determining the number of days in the period beginning on the date a clinical investigation on humans involving the device was begun and ending on the date an application was initially submitted with respect to the device under section 515 of the Federal Food, Drug, and Cosmetic Act = 2338 days (between 11/14/91 and 4/8/98).

(b) Determining the number of days in the period beginning on the date the application was initially submitted with respect to the device under section 515 of

the Federal Food, Drug, and Cosmetic Act, and ending on the date that such application was approved = 1268 days (4/8/98 to 1/29/01 and 2/20/01 to 10/17/01).

(c) Adding the results of paragraphs (a) and (b) = 3606 days (2338 + 1268).

(d) Determining the number of days in the periods of paragraphs (a) and (b) which were on and before the date on which the patent issued = 720 days (11/14/91 to 11/2/93).

(e) Determining the number of days in the periods of paragraphs (a) and (b) in which applicant did not act with due diligence = 0 days

(f) Determining one half the number of days remaining in the period defined by paragraph (a) after that period is reduced in accordance with paragraphs (d) and (e) = 809 days ((2338 days - 720 days)/2).

(g) Subtracting the number of days determined in paragraphs (d), (e) and (f) from the number of days determined in paragraph (c) = 2077 days (3606 - 720 - 809).

(h) Adding the number of days determined in paragraph (g) to the original term of the Patent (November 2, 2010) as shortened by any terminal disclaimer = July 10, 2016.

(i) Adding 14 years to the date of approval of the application under section 515 of the Federal Food, Drug, and Cosmetic Act (October 17, 2001) = October 17, 2015.

(j) Comparing the dates obtained in paragraphs (h) and (i) and selecting the earlier date = October 17, 2015.

(k) Adding 5 years to the original expiration date of the Patent (November 2, 2010) or earlier date set by terminal disclaimer = November 2, 2015.

(l) Comparing the dates obtained in paragraphs (j) and (k) and selecting the earlier date = October 17, 2015.

In the opinion of applicant, therefore, the term of the Patent should be extended to October 17, 2015.

(13) STATEMENT THAT APPLICANT ACKNOWLEDGES A DUTY TO DISCLOSE TO THE COMMISSIONER OF PATENTS AND TRADEMARKS AND THE SECRETARY OF HEALTH AND HUMAN SERVICES ANY INFORMATION MATERIAL TO THE DETERMINATION OF ENTITLEMENT OF EXTENSION SOUGHT

Applicant hereby acknowledges a duty to disclose to the Commissioner of Patents and Trademarks and the Secretary of Health and Human Services any information which is material to the determination of entitlement to the extension sought.

(14) PRESCRIBED FEE

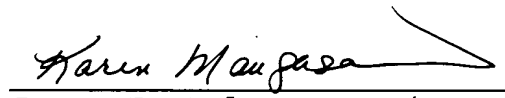
A check in the amount of $1,120.00 in payment of the fee pursuant to 37 C.F.R. § 1.20(j) is enclosed herewith. The Director is authorized to charge any additional fees to Deposit Account No. 06-1075.

(15) CORRESPONDENCE ADDRESS

Please forward all inquiries and correspondence relating to this application for patent term extension to:

James F. Haley, Jr.
Fish & Neave
1251 Avenue of the Americas
New York, New York 10020-1104
Tel: (212) 596-9000
FAX: (212) 596-9090

Respectfully submitted,



James F. Haley, Jr. (Reg. No. 27,794)
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SUPPLEMENTARY INFORMATION: The Drug Price Competition and Patent Term Restoration Act of 1984 (Public Law 98–417) and the Generic Animal Drug and Patent Term Restoration Act (Public Law 100–670) generally provide that a patent may be extended for a period of up to 5 years so long as the patented item (human drug product, animal drug product, medical device, food additive, or color additive) was subject to regulatory review by FDA before the item was marketed. Under these acts, a product's regulatory review period forms the basis for determining the amount of extension an applicant may receive.

A regulatory review period consists of two periods of time: A testing phase and an approval phase. For medical devices, the testing phase begins with a clinical investigation of the device and runs until the approval phase begins. The approval phase starts with the initial submission of an application to market the device and continues until permission to market the device is granted. Although only a portion of a regulatory review period may count toward the actual amount of extension that the Director of Patents and Trademarks may award (half the testing phase must be subtracted as well as any time that may have occurred before the patent was issued), FDA's determination of the length of a regulatory review period for a medical device will include all of the testing phase and approval phase as specified in 35 U.S.C. 156(g)(3)(B).

FDA recently approved for marketing the medical device GYNECARE INTERGEL. GYNECARE INTERGEL is indicated for use in patients undergoing open, conservative gynecologic surgery as an adjunct to good surgical technique to reduce postsurgical adhesions. Subsequent to this approval, the Patent and Trademark Office received a patent term restoration application for GYNECARE INTERGEL (U.S. Patent No. 5,532,221) from Lifecore Medical, Inc., and the Patent and Trademark Office requested FDA's assistance in determining this patent's eligibility for patent term restoration. In a letter dated October 31, 2001, FDA advised the Patent and Trademark Office that this medical device had undergone a regulatory review period and that the approval of GYNECARE INTERGEL represented the first permitted commercial marketing or use of the product. Thereafter, the Patent and Trademark Office requested that FDA determine the product's regulatory review period.

FDA has determined that the applicable regulatory review period for GYNECARE INTERGEL is 2,438 days. Of this time, 1,453 days occurred during the testing phase of the regulatory review period, while 985 days occurred during the approval phase. These periods of time were derived from the following dates:

1. *The date a clinical investigation involving this device was begun*: March 17, 1995. FDA has verified the applicant's claim that the date the investigational device exemption (IDE) required under section 520(g) of the Federal Food, Drug, and Cosmetic Act (the act) (21 U.S.C. 360j(g)) for human tests to begin became effective March 17, 1995.

2. *The date an application was initially submitted with respect to the device under section 515 of the act (21 U.S.C. 360e)*: March 8, 1999. The applicant claims March 5, 1999, as the date the premarket approval application (PMA) FOR GYNECARE INTERGEL (PMA P990015) was initially submitted. However, FDA records indicate that PMA P990015 was submitted on March 8, 1999.

3. *The date the application was approved*: November 16, 2001. FDA has verified the applicant's claim that PMA P990015 was approved on November 16, 2001.

This determination of the regulatory review period establishes the maximum potential length of a patent extension. However, the U.S. Patent and Trademark Office applies several statutory limitations in its calculations of the actual period for patent extension. In its application for patent extension, this applicant seeks 867 days of patent term extension.

Anyone with knowledge that any of the dates as published are incorrect may submit to the Dockets Management Branch (see **ADDRESSES**) written or electronic comments and ask for a redetermination by June 2, 2003. Furthermore, any interested person may petition FDA for a determination regarding whether the applicant for extension acted with due diligence during the regulatory review period by Septemebr 29, 2003. To meet its burden, the petition must contain sufficient facts to merit an FDA investigation. (See H. Rept. 857, part 1, 98th Cong., 2d sess., pp. 41–42, 1984.) Petitions should be in the format specified in 21 CFR 10.30.

Comments and petitions should be submitted to the Dockets Management Branch. Three copies of any information are to be submitted, except that individuals may submit one copy. Comments are to be identified with the docket number found in brackets in the heading of this document. Comments and petitions may be seen in the Dockets Management Branch between 9 a.m. and 4 p.m., Monday through Friday.

Dated: February 6, 2003.

Jane A. Axelrad,

Associate Director for Policy, Center for Drug Evaluation and Research.

[FR Doc. 03–7819 Filed 4–1–03; 8:45 am]

BILLING CODE 4160–01–S

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration

[Docket No. 02E–0147]

Determination of Regulatory Review Period for Purposes of Patent Extension; OP–1 IMPLANT

AGENCY: Food and Drug Administration, HHS.

ACTION: Notice.

SUMMARY: The Food and Drug Administration (FDA) has determined the regulatory review period for OP–1 IMPLANT and is publishing this notice of that determination as required by law. FDA has made the determination because of the submission of an application to the Director of Patents and Trademarks, Department of Commerce, for the extension of a patent which claims that medical device.

ADDRESSES: Submit written comments and petitions to the Dockets Management Branch (HFA–305), Food and Drug Administration, 5630 Fishers Lane, rm. 1061, Rockville, MD 20852. Submit electronic comments to *http://www.fda.gov/dockets/ecomments.*

FOR FURTHER INFORMATION CONTACT: Claudia Grillo, Office of Regulatory Policy (HFD–013), Food and Drug Administration, 5600 Fishers Lane, Rockville, MD 20857, 301–827–3460.

SUPPLEMENTARY INFORMATION: The Drug Price Competition and Patent Term Restoration Act of 1984 (Public Law 98–417) and the Generic Animal Drug and Patent Term Restoration Act (Public Law 100–670) generally provide that a patent may be extended for a period of up to 5 years so long as the patented item (human drug product, animal drug product, medical device, food additive, or color additive) was subject to regulatory review by FDA before the item was marketed. Under these acts, a product's regulatory review period forms the basis for determining the amount of extension an applicant may receive.

A regulatory review period consists of two periods of time: A testing phase and an approval phase. For medical devices,

the testing phase begins with a clinical investigation of the device and runs until the approval phase begins. The approval phase starts with the initial submission of an application to market the device and continues until permission to market the device is granted. Although only a portion of a regulatory review period may count toward the actual amount of extension that the Director of Patents and Trademarks may award (half the testing phase must be subtracted as well as any time that may have occurred before the patent was issued), FDA's determination of the length of a regulatory review period for a medical device will include all of the testing phase and approval phase as specified in 35 U.S.C. 156(g)(3)(B).

FDA recently approved for marketing the medical device OP-1 IMPLANT. OP-1 IMPLANT is indicated for use as an alternative to the patient's own bone (autograft) in recalcitrant long bone nonunions where autograft is unfeasible and alternative treatments have failed. Subsequent to this approval, the Patent and Trademark Office received a patent term restoration application for OP-1 IMPLANT (U.S. Patent No. 5,258,494) from Stryker Corp., and the Patent and Trademark Office requested FDA's assistance in determining this patent's eligibility for patent term restoration. In a letter dated October 31, 2001, FDA advised the Patent and Trademark Office that this medical device had undergone a regulatory review period and that the approval of OP-1 IMPLANT represented the first permitted commercial marketing or use of the product. Thereafter, the Patent and Trademark Office requested that FDA determine the product's regulatory review period.

FDA has determined that the applicable regulatory review period for OP-1 IMPLANT is 3,627 days. Of this time, 3,485 days occurred during the testing phase of the regulatory review period, while 142 days occurred during the approval phase. These periods of time were derived from the following dates:

1. *The date a clinical investigation involving this device was begun*: November 14, 1991. FDA has verified the applicant's claim that the date the investigational device exemption (IDE) required under section 520(g) of the Federal Food, Drug, and Cosmetic Act (the act) (21 U.S.C. 360j(g)) for human tests to begin became effective November 14, 1991.

2. *The date the application was initially submitted with respect to the device under section 515 of the act (21 U.S.C. 360e)*: May 29, 2001. The applicant claims May 25, 2001, as the date the premarket approval application (PMA) for OP-1 IMPLANT (PMA HO10002/A01) was initially submitted. However, FDA records indicate that PMA HO10002/A01 was submitted on May 29, 2001.

3. *The date the application was approved*: October 17, 2001. FDA has verified the applicant's claim that PMA HO10002/A01 was approved on October 17, 2001.

This determination of the regulatory review period establishes the maximum potential length of a patent extension. However, the U.S. Patent and Trademark Office applies several statutory limitations in its calculations of the actual period for patent extension. In its application for patent extension, this applicant seeks 1,837 days of patent term extension.

Anyone with knowledge that any of the dates as published is incorrect may by submit to the Dockets Management Branch (see **ADDRESSES**) written or electronic comments and ask for a redetermination by June 2, 2003. Furthermore, any interested person may petition FDA for a determination regarding whether the applicant for extension acted with due diligence during the regulatory review period by September 29, 2003. To meet its burden, the petition must contain sufficient facts to merit an FDA investigation. (See H. Rept. 857, part 1, 98th Cong., 2d sess., pp. 41–42, 1984.) Petitions should be in the format specified in 21 CFR 10.30.

Comments and petitions should be submitted to the Dockets Management Branch. Three copies of any information are to be submitted, except that individuals may submit one copy. Comments are to be identified with the docket number found in brackets in the heading of this document. Comments and petitions may be seen in the Dockets Management Branch between 9 a.m. and 4 p.m., Monday through Friday.

Dated: February 7, 2003.

Jane A. Axelrad,

Associate Director for Policy, Center for Drug Evaluation and Research.

[FR Doc. 03-7820 Filed 4-1-03; 8:45 am]

BILLING CODE 4160-01-S

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration

[Docket No. 03D-0111]

Draft Guidance for Federal Agencies and State and Local Governments; Potassium Iodide Shelf Life Extension; Availability

AGENCY: Food and Drug Administration, HHS.

ACTION: Notice.

SUMMARY: The Food and Drug Administration (FDA) is announcing the availability of a draft guidance for Federal agencies and State and local governments entitled "Potassium Iodide Shelf Life Extension." This document is intended to provide guidance to Federal agencies and to State and local governments on testing to extend the shelf life of stockpiled potassium iodide (KI) tablets. The draft guidance discusses FDA recommendations on the requisite testing for KI tablet shelf life extensions, the qualifications of laboratories suitable to conduct the tests, and issues regarding notification of holders of stockpiled KI tablets as well as end users about changes to batch shelf life once testing has been successfully conducted.

DATES: Submit written or electronic comments on the draft guidance by June 2, 2003. General comments on agency guidance documents are welcome at any time.

ADDRESSES: Submit written requests for single copies of this draft guidance to the Division of Drug Information (HFD-240), Center for Drug Evaluation and Research, Food and Drug Administration, 5600 Fishers Lane, Rockville, MD 20857. Send one self-addressed adhesive label to assist that office in processing your requests. Submit written comments on the draft guidance to the Dockets Management Branch (HFA-305), Food and Drug Administration, 5630 Fishers Lane, rm. 1061, Rockville, MD 20852. Submit electronic comments to *http://www.fda.gov/dockets/ecomments*. See the **SUPPLEMENTARY INFORMATION** section for electronic access to the draft guidance document.

FOR FURTHER INFORMATION CONTACT: Richard Adams, Center for Drug Evaluation and Research (HFD-643), Food and Drug Administration, 7500 Standish Pl., Rockville, MD 20855, 301-827-5849.

SUPPLEMENTARY INFORMATION:

Trademark Electronic Search System(Tess)

TESS was last updated on Fri Feb 25 04:28:41 EST 2005

Check Status *(TARR contains current status, correspondence address and attorney of record for this mark. Use the "Back" button of the Internet Browser to return to TESS)*

Typed Drawing

Word Mark	**OP-1**
Goods and Services	IC 005. US 006 018 044 046 051 052. G & S: osteogenic graft material for skeletal repair and regeneration. FIRST USE: 20011119. FIRST USE IN COMMERCE: 20011119
Mark Drawing Code	(1) TYPED DRAWING
Serial Number	75637319
Filing Date	February 10, 1999
Current Filing Basis	1A
Original Filing Basis	1B
Published for Opposition	November 30, 1999
Registration Number	2663601
Registration Date	December 17, 2002
Owner	(REGISTRANT) Stryker Corporation CORPORATION MICHIGAN 190 North Main Street Natick MASSACHUSETTS 01760
Attorney of Record	Robert R. Pierce
Type of Mark	TRADEMARK
Register	PRINCIPAL

Live/Dead Indicator LIVE

From PMA modular submission to PMA dated May 21, 1998. Volume 1, Section B.1.b.

"B. 1. b Osteogenic Protein-1 (OP-1)

General Introduction

OP-1 stimulates the development of bone within the matrix established by the collagen. It is part of a family of proteins involved in bone formation which are found naturally in human bone. OP-1 is osteoinductive and functions in the bone formation process as the initiator of the biological cascade recruiting mesenchymal stem cells to the implant site where they differentiate and stimulate bone cell proliferation.

Background Information

OP-1, also called bone morphogenetic protein-7 (BMP-7) was originally isolated from bone based on its ability to induce new bone formation *in vivo* when associated with an appropriate physical support or matrix for cellular attachment.

OP-1 is manufactured by purification of a protein expressed by an engineered Chinese hamster ovary (CHO) cell line, DXB11. [Claim 1 and 11] This cell line was engineered for expression of the human OP-1 protein under control of Cytomegalovirus major immediate early (CMV-MIE) promoter.

OP-1 Structure

Recombinant OP-1 is synthesized in a precursor form as a 431 amino acid protein which is subsequently processed to a 139 amino acid mature form (see Volume 5). Figure 1. B. 5 shows the amino acid sequence encoding the mature
OP-1.

The active form of OP-1 consists of two 139 amino acid monomers connected by a disulfide bond.[Claim 1 and 16] The approximate molecular weight of the monomer form is 18 kilo Daltons. Each monomer contains a single N-linked glycosylation site Asn 80.

Figure 1. B. 5 : Amino Acid Sequence of the mature OP-1.

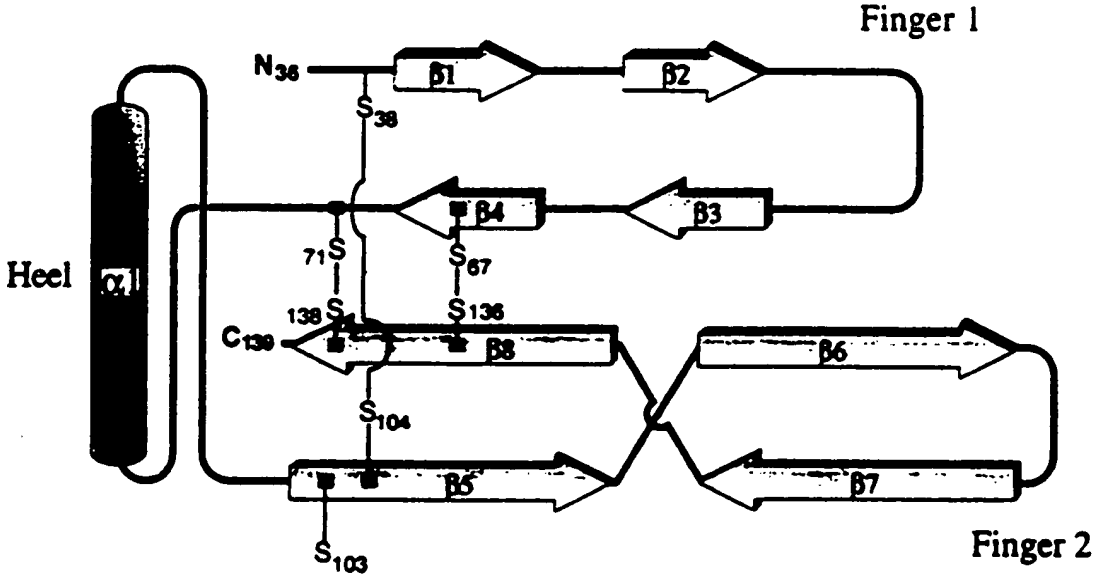
1									***										20
Ser	*Thr*	*Gly*	*Ser*	*Lys*	*Gln*	*Arg*	*Ser*	*Gln*	*Asn*	*Arg*	*Ser*	*Lys*	*Thr*	*Pro*	*Lys*	*Asn*	*Gln*	*Glu*	*Ala*
21								***	*30*										*40*
Leu	*Arg*	*Met*	*Ala*	*Asn*	*Val*	*Ala*	*Glu*	*Asn*	*Ser*	*Ser*	*Ser*	*Asp*	*Gln*	*Arg*	*Gln*	*Ala*	*Cys*	*Lys*	*Lys*
41									*50*										*60*
His	*Glu*	*Leu*	*Tyr*	*Val*	*Ser*	*Phe*	*Arg*	*Asp*	*Leu*	*Gly*	*Trp*	*Gln*	*Asp*	*Trp*	*Ile*	*Ile*	*Ala*	*Pro*	*Glu*
61									*70*										***
Gly	*Tyr*	*Ala*	*Ala*	*Tyr*	*Tyr*	*Cys*	*Glu*	*Gly*	*Glu*	*Cys*	*Ala*	*Phe*	*Pro*	*Leu*	*Asn*	*Ser*	*Tyr*	*Met*	*Asn*
81									*90*										*100*
Ala	*Thr*	*Asn*	*His*	*Ala*	*Ile*	*Val*	*Gln*	*Thr*	*Leu*	*Val*	*His*	*Phe*	*Ile*	*Asn*	*Pro*	*Glu*	*Thr*	*Val*	*Pro*
101									*110*										*120*
Lys	*Pro*	*Cys*	*Cys*	*Ala*	*Pro*	*Thr*	*Gln*	*Leu*	*Asn*	*Ala*	*Ile*	*Ser*	*Val*	*Leu*	*Tyr*	*Phe*	*Asp*	*Asp*	*Ser*
121									*130*									*139*	
Ser	**Asn**	**Val**	**Ile**	**Leu**	**Lys**	**Lys**	**Tyr**	**Arg**	**Asn**	**Met**	**Val**	**Val**	**Arg**	**Ala**	**Cys**	**Gly**	**Cys**	**His**	

*Potential N-glycosylation sites are shown by ***.*

Monomer

The overall OP-1 monomer fold has approximate dimensions of 60 Å X 20 Å X 15 Å. It has been compared to a "left hand". Each monomer of OP-1 contains seven cysteines. Six of the seven cysteines are involved in intrachain disulfide bonds with each monomeric unit and the remaining cysteine participates in an intermolecular disulfide bond to form the dimer. The arrangement of disulfide bonds has been termed the cysteine knot.[7]

Figure 1. B. 6 : Schematic drawing of the OP-1 monomer fold. [7]



This OP-1 cysteine knot constitutes the core of the monomer and consists of three disulfide bonds; two, Cys-67-Cys-136 and Cys-71-Cys-138, form a ring through which the third, Cys-38-Cys-104, passes. The four strands of antiparallel β-sheet, which emanate from the knot, form two finger-like projections. An α-helix, located on the opposite end of the knot, lies perpendicular to the axis of the two fingers thereby forming the heel of the hand. The N terminus that corresponds to the thumb of the hand is unresolved in Figure 1. B. 6. The N-terminal region is not stabilized by a disulfide bond.

The finger 1 region of OP-1 is an antiparallel β-sheet containing a 13-residue Ω loop. The conformation of the Ω loop orients six nonpolar residues so they can contribute to a solvent-inaccessible interface with finger 2.

The only α-helix in the monomer is located between the third and fifth Cys residues (Cys-71 and Cys-104). This helix extends for 3.5 turns from residues Thr-82 to Ile-94, is amphipathic, and contains a number of hydrophobic residues that make contact in the dimer with residues from finger 1 and finger 2 of the monomer.

Finger 2 is the second antiparallel β-sheet in OP-1. The polypeptide chain reverses direction between segments β6 and β7. Residues Arg-129 to Val-132, located between segments β7 and β8, form a peptide bridge that crosses over the C-terminal end of strand β5 and produces a 180° twist in the finger 2 antiparallel β-structure.

Within the monomer, finger 2 makes intrachain contacts with finger 1 by contributing aromatic residues Tyr-116, Phe-117, and Tyr-128 and aliphatic residues Val-114, Leu-115, Val-123, Met-131, and Val-133 to a solvent-inaccessible interface.

Dimer

The OP-1 dimer is formed by the association of the heel region α-helix of one monomer with the finger regions of the other monomer and is stabilized by a single interchain disulfide bond involving Cys-103. Formation of this disulfide bond brings the cystine knot regions close together with the monomers oriented such that the molecular twofold axis passes between and parallel to the rings of the knots and perpendicular to the axes of the fingers. Figure 1. B. 7 shows the 3-dimensional structure of OP-1."

B

stryker BIOTECH

OP-1 IMPLANT PACKAGE INSERT

HUMANITARIAN DEVICE. OP-1 Implant is authorized by Federal law for use as an alternative to autograft in recalcitrant long bone nonunions where use of autograft is unfeasible and alternative treatments have failed. The effectiveness of this device for this use has not been demonstrated.

Caution: Federal law restricts this device to sale by or on the order of a physician.

PRODUCT DESCRIPTION:
OP-1 Implant is an osteoinductive and osteoconductive bone graft material. It is supplied in a glass vial containing one gram of the device in the form of a sterile dry powder comprised of recombinant human Osteogenic Protein 1 (OP-1 or BMP-7) and bovine bone collagen.

Self-adhesive patient labels indicating the lot number of the implant are provided for the user's convenience.

STORAGE CONDITIONS:
Store OP-1 Implant at 2-8 °C.

INDICATIONS:
OP-1 Implant is indicated for use as an alternative to autograft in recalcitrant long bone nonunions where use of autograft is unfeasible and alternative treatments have failed.

CONTRAINDICATIONS:
- OP-1 Implant should not be used to treat patients who have a known hypersensitivity to the active substance or to collagen.
- OP-1 Implant should not be applied at the site of a resected tumor which is at or near the vicinity of the defect/fracture or in patients with a history of malignancy.
- OP-1 Implant should not be administered to patients who are skeletally immature (<18 years of age or no radiographic evidence of closure of epiphyses).
- OP-1 Implant should not be administered to pregnant women. The potential effects of OP-1 treatment on the human fetus have not been evaluated. Studies in rats injected with high doses of OP-1 have shown that small amounts of OP-1 will cross the placental barrier.

WARNINGS:
- Women of childbearing potential should be advised that antibody formation to OP-1 and its influence on fetal development have not been assessed. 38% of patients treated with OP-1 Implant and 13% of patients treated with autograft develop antibodies. The effect of maternal antibodies to OP-1 on the unborn fetus is unknown, both when the antibodies are detected during the first year following treatment and later, when the antibodies may not be detectable. Studies in genetically altered mice indicate that OP-1 is critical for fetal development and that lack of OP-1 activity, as might be induced by antibody, may cause neonatal death or birth defects.
- Women of childbearing potential should be advised to use contraception for one year following treatment with OP-1 Implant.
- The maximum human dose should not exceed 2 vials. In clinical studies treating nonunions requiring more than 2 vials, there was a higher incidence of failure.
- OP-1 Implant has no biomechanical strength to support fixation without a shared loading/stabilization adjunct (i.e., cast, instrumentation, etc.) in long bones. The following fixation methods have been utilized in clinical trials studying OP-1 Implant: cast/brace, external fixation, intramedullary rod and internal plate.
- Localized ectopic or heterotopic bone formation may occur outside of the treatment site.

PRECAUTIONS:
- Clinical studies using OP-1 Implant were performed in patients with nonunions resulting from trauma. There are no data regarding the use of OP-1 Implant in patients with nonunions resulting from bone diseases.
- OP-1 Implant may cause an immune reaction in some patients. The safety or probable benefit of OP-1 Implant in patients with autoimmune disease has not been demonstrated.
- The effect of radiation therapy, chemotherapy, immunosuppressive or steroid therapy on the probable benefit of OP-1 Implant is not known.
- There are no data on the excretion of OP-1 in the breast milk of patients who are nursing.
- OP-1 is important in the development of the kidney. Studies have not been performed to examine the neutralizing capacity of antibodies to OP-1 or their effect in patients with impaired renal function.
- IMMUNOGENICITY: As with all therapeutic proteins, there is a potential for immune responses to be generated against components of the OP-1 Implant. In the Tibial Nonunion clinical study, antibodies were detected to OP-1 (BMP-7) by an ELISA assay in 23/61 (38%) OP-1 treated patients and 8/61 (13%) autograft treated patients and confirmed by Western Blot analysis. The neutralizing capacity of these antibodies was not assessed. The significance of these antibodies is not known. The incidence of antibody detection is highly dependent on the sensitivity and specificity of the assay. The sensitivity of the antibody assay has not been adequately assessed and the actual incidence of antibodies could be higher. Additionally, the incidence of antibody detection may be influenced by several factors including sample handling, concomitant medications, and underlying disease. For these reasons, comparison of the incidence of antibodies to OP-1 Implant with the incidence of antibodies to other products may be misleading.
- A two year rat bioassay, in which approximately 17.5-70 times the equivalent maximum human dose of 2 vials of OP-1 Implant was placed under the skin, produced more cancer growths at the site of implantation of the OP-1 Implant compared to rats that had no OP-1 Implant. It is believed that this may be due to the Oppenheimer Solid State Tumor Effect, the formation of tumors at the site of implantation of inert objects under the skin in rats. This effect has not been reported in humans. Additional studies are ongoing to examine the effect of OP-1 on the growth of pre-existing tumors.
- Take care to ensure that OP-1 Implant will be contained by viable hard and soft tissue structures. Obtain adequate hemostasis before implanting OP-1 Implant to prevent the product from being displaced.
- Inadequate vascularity in the surrounding tissues may diminish the probable benefit of OP-1 Implant. Make every effort to surround the product with viable tissue.
- For single use only. Do not re-use OP-1 Implant. Discard unused product and use a new device for subsequent applications.
- Prior to use, inspect the packaging, vial and stopper for visible damage. If damage is visible, don't use the product. Retain the packaging and vial, and contact a Stryker Biotech representative.
- Do not use after the printed expiration date on the label.

ADVERSE EVENTS:
The following table is from two multicenter studies of OP-1 Implant in patients with long bone nonunions. Reported in the table are adverse events relevant to an orthopaedic procedure occurring in >1% of the total treated patients. Other less frequent and related events are listed in Table 1 below. No serious adverse events were attributed to the use of OP-1 Implant.

Table 1 - Summary of Adverse Events for All Patients in the Two Long Bone Nonunion Studies

Adverse Event Description	Tibial Nonunion Study		Long Bone Nonunion Study
	OP-1 Implant n=61	Autograft n=61	OP-1 Implant n=29
Musculoskeletal			
Hardware Compression	28/61	40/61	6/29
Nonunion	7/61	4/61	5/29
Osteomyelitis	6/61	15/61	7/29
Injury as a Result of Fall	3/61	3/61	2/29
Malunion	3/61	0/61	1/29
Hardware removal	2/61	1/61	0/29
Tendonitis (patellar, Achilles)	2/61	1/61	0/29
Contracture	1/61	3/61	1/29
Fracture (other)	1/61	3/61	0/29
Fracture (tibia, fibula)	1/61	3/61	1/29
Skin and Wound			
Wound infection	18/61	14/61	5/29
Local inflammation, rash, redness, itching	12/61	10/61	0/29
Swelling (ankle, foot, leg)	7/61	8/61	2/29
Blisters, skin abrasions	5/61	0/61	0/29
Neural			
Pain (ankle, knee, leg)	27/61	22/61	12/29
Neuralgia (numbness)	6/61	6/61	3/29
Pain (other)	3/61	3/61	3/29
Nerve injury	2/61	2/61	0/29
Cardiovascular			
Hematoma	4/61	8/61	3/29
Anemia	4/61	5/61	1/29
Gastro-Intestinal			
Nausea, vomiting	18/61	19/61	3/29
Gastro-intestinal upset (indigestion, constipation, diarrhea)	7/61	5/61	1/29
Systemic and Other Complications			
Fever	31/61	29/61	0/29
Normal Surgical Complications	10/61	8/61	0/29
Drug Allergy (morphine, antibiotics)	2/61	5/61	1/29

Other events include: amputation of toe, aortocoronary bypass with valve replacement, arthritis, arthroscopy, arthrosis, athlete's foot, bruising, burning sensation, cardiac complications following surgery, chondrectomy, chondromalacia, cold symptoms/upper respiratory infection, death-unrelated causes, depression, dizziness, ear infection, fatigue, gangrene, headache/migraine, incontinence, insomnia, meniscal tear, muscle spasm, muscular herniation, myositis ossificans, nosebleeds, pancreatitis, peptic ulcer, plantar fascial fibromatosis, post operative bleeding, sciatica, skin graft, short term memory loss, shortness of breath, slow or decreased urination, stiffness, sweating, thrombophlebitis, thrombosis, urinary tract infection, weight loss, wound dehiscence, yeast infection.

24

Five (8%) OP-1 Implant patients reported 6 treatment related medical events, including persistent nonunion (3), erythema/swelling (2) and drainage (1).

Adverse event data has been collected from over 500 domestic and international patients treated with OP-1. Table 2 describes the incidence of cancer reported in these patients.

Table 2 – Incidence of Cancer in Patients Treated with OP-1 Worldwide

Cancer Type	Age	Sex	Time of Event Post-treatment with OP-1	Outcome
Pancreatic tumor with multiple metastases	83yrs.	Male	3 Months	Death
Gastric carcinoma	79yrs.	Male	9 Months	Recovered
Mantle cell lymphoma	76yrs.	Female	29 Months	Death
Right occipital basal cell carcinoma (non-healing forehead lesion)	60yrs.	Male	11 Months	Recovered
Recurrence of Chondrosarcoma	43yrs.	Female	6 weeks*	Death

* Treating physician believes recurrence may have presented on thallium scan prior to treatment with OP-1.

Five patients reported the occurrence of cancer. Four of the 5 events reported were non-osseous cancers occurring in elderly patients. A fifth event of recurring chondrosarcoma was reported in a patient with a history of chondrosarcoma. Recurrence and disease progression were considered normal for this type of cancer. The incidence of cancer in patients treated with OP-1 is less than 1% and is within the range of cancer occurrence in the general populations of the U.S. and Australia (the countries in which most patients were treated).

Eight (1.6%) out of more than 500 patients treated with OP-1 experienced 10 events related to urinary or renal systems. All 10 events were considered by the treating physicians as unrelated to study treatment and were mild to moderate in severity. No severe adverse events of this nature were reported. Events included urinary tract infection (5), slow urination (1), decreased urine output (1), urinary retention (1) and retrograde ejaculation (2). Many of these events were reported immediately post-treatment and can be attributed to catheterization during and after surgery.

PREPARATION FOR USE:

OP-1 Implant is intended to be reconstituted with sterile Sodium Chloride (NaCl) Injection, 0.9%, USP solution (saline).

1. Using sterile technique, remove the vial from its packaging.
2. Lift the plastic flip-top and remove the crimp from the vial.

Warning: Handle the crimp with care. The edges of the crimp are sharp and may cut or damage gloves.

3. Aligning your thumb with the internal gap of the stopper, pry up the edge of the stopper. Once the vacuum is broken, remove the vial stopper while holding the vial upright to prevent loss of product.

Warning: Do not insert a needle through the stopper. Puncture of the stopper with a needle may result in particles of stopper material contaminating the OP-1 Implant.

4. Utilizing a sterile syringe, carefully add 2-3 cc of sterile saline to OP-1 Implant in the vial. Begin with 2 cc and add saline to desired consistency. Use of more than 3 cc will result in a less cohesive product which will be difficult to handle.
5. Mix the saline with the product in the vial using a sterile spatula or curette.
6. The product will expand to a maximum volume (~4cc) within 2 minutes. Use the product promptly after reconstituting with saline.

RECOMMENDED TECHNIQUE:

1. Debride fibrous, necrotic or sclerotic tissue and, when appropriate, decorticate bone so that OP-1 Implant will directly contact viable osseous tissue.
2. Provide adequate hemostasis to ensure that the material stays at the surgical site. Irrigate the surgical site as necessary, prior to placement of OP-1 Implant. Where practical, surgical manipulations to the site should be completed prior to device implantation.
3. Remove the reconstituted OP-1 Implant from the vial with a sterile instrument such as a spatula or curette.
4. Apply OP-1 Implant to the prepared osseous tissue site. The amount of material used should approximate the size of the bone defect.

   Warning: Do not use suction or irrigation directly at the implant site as this may remove particles of OP-1 Implant. Remove excess fluid by suctioning adjacent to the implant site or carefully blotting the area with a sterile sponge.
5. Close soft tissues around the defect containing OP-1 Implant using suture material of choice. Closure is critical for containment and maintenance of OP-1 Implant particles in the area of the defect.
6. After closure of the soft tissue around the defect, irrigate field, if necessary, to remove any stray particles.

Do not place a drain directly in the implant site. Place it subcutaneously if possible.

CLINICAL EXPERIENCE:

Clinical experience with OP-1 Implant in the intended indication is summarized below. In a multicenter Tibial Nonunion Study, a subset of 14 patients with prior failed autograft were treated with OP-1 Implant. In a second multicenter Long Bone Treatment Study, 10 patients with long bone nonunions having prior failed autograft were treated with OP-1 Implant. Results are shown in Tables 3 and 4 below:

Table 3 - Tibial Nonunion Study Results– Prior autograft patients only

Analysis at 9 Months	OP-1 Implant N=14	Autograft N=13
Overall	7/14	11/13
Clinical (Pain and Function)	12/14	12/13
Radiographic (Bridging in 3 views)	8/14	12/13

Table 4 - Long Bone Treatment Study Results

Analysis at 9 Months	OP-1 Implant N=10
Overall	1/10
Clinical (Pain and Function)	7/10
Radiographic (Bridging in 3/4 cortices)	2/10

Manufactured by

stryker BIOTECH

Registration # 9045977
35 South Street
Hopkinton, MA 01748 USA
Phone (508) 416-5200
www.op1.com

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Stryker Corporation
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Kalamazoo, MI 49003

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
9200 Corporate Boulevard
Rockville MD 20850

OCT 17 2001

Amy J. LaForte, Ph.D.
Director, Regulatory Affairs
Stryker Biotech
35 South Street
Hopkinton, MA 01748

Re: H010002
OP-1™ Implant
Filed: May 29, 2001
Amended: May 29, June 28, July 5 and 23, and October 9, 11, and 17, 2001

Dear Dr. LaForte:

The Center for Devices and Radiological Health (CDRH) of the Food and Drug Administration (FDA) has completed its review of your humanitarian device exemption (HDE) application for the OP-1™ Implant. This device is indicated for use as an alternative to autograft in recalcitrant long bone nonunions where use of autograft is unfeasible and alternative treatments have failed. CDRH is pleased to inform you that your HDE is approved subject to the enclosed "Conditions of Approval." You may begin commercial distribution of the device after you have submitted an amendment to this HDE with copies of the approved labeling in final printed form.

In addition to the postapproval requirements in the enclosure, you have agreed to provide:

1. a preclinical plan for assessing the effects of OP-1™ on tumor promotion;
2. a plan for addressing the preclinical and clinical immunological commitments that you have made; and
3. a plan to collect pregnancy outcomes that will be reported in your annual report.

Please submit the study plans for the first two items within 45 days of receipt of this letter. You may submit your response to the third item within 3-6 months of receipt of this letter. The results of these postapproval studies may require modifications to be made in the labeling (via a supplement) when the studies are completed.

The sale, distribution, and use of this device are limited to prescription use in accordance with 21 CFR 801.109 within the meaning of section 520(e) of the Federal Food, Drug, and Cosmetic Act (the act) under the authority of section 515(d)(1)(B)(ii) of the act. In addition, in order to ensure the safe use of the device, FDA has further restricted the device within the meaning of section 520(e) of the act under the authority of section 515(d)(1)(B)(ii) of the act insofar as the sale, distribution, and use must not violate sections 502(q) and (r) of the act.

FDA wishes to remind you that failure to comply with the conditions of approval invalidates this approval order. Commercial distribution of a device that is not in compliance with these conditions is a violation of the act.

CDRH will notify the public of its decision to approve your HDE by making available a summary of the safety and probable benefit of the device upon which the approval was based. The information can be found on the FDA CDRH Internet HomePage located at http://www.fda.gov/cdrh/ode/hdeinfo.html. Written requests for this information can also be made to the Dockets Management Branch (HFA-305), Food and Drug Administration, 5630 Fishers Lane, Rm. 1061, Rockville, MD 20852. The written request should include the HDE number or docket number. Within 30 days from the date that this information is placed on the Internet, any interested person may seek review of this decision by requesting an opportunity for administrative review, either through a hearing or review by an independent advisory committee, under section 515(g) of the act.

You are reminded that, as soon as possible and before commercial distribution of your device, you must submit an amendment to this HDE submission with copies of all approved labeling in final printed form. As part of our reengineering effort, the Office of Device Evaluation is piloting a new process for review of final printed labeling. The labeling will not routinely be reviewed by FDA staff when HDE applicants include with their submission of the final printed labeling a cover letter stating that the final printed labeling is identical to the labeling approved in draft form. If the final printed labeling is not identical, any changes from the final draft labeling should be highlighted and explained in the amendment. Please see the CDRH Pilot for Review of Final Printed Labeling document at http://www.fda.gov/cdrh/pmat/pilotpmat.html for further details.

Any information to be submitted to FDA regarding this HDE should be submitted in triplicate, unless otherwise specified, to the address below and should reference the above HDE number to facilitate processing:

Document Mail Center (HFZ-401)
Office of Device Evaluation
Center for Devices and Radiological Health
Food and Drug Administration
9200 Corporate Blvd.
Rockville, Maryland 20850

If you have any questions concerning this approval order, please contact Ms. Jan C. Callaway at (301) 594-2018.

Sincerely yours,

Daniel G. Schultz, M.D.
Deputy Director for Clinical and Review Policy
Office of Device Evaluation
Center for Devices and Radiological Health

Enclosure
"Conditions of Approval"

CONDITIONS OF APPROVAL FOR AN HDE

I. APPROVED LABELING

As soon as possible and before commercial distribution of the device, the holder of an HDE should submit three copies of the approved labeling in final printed form as an amendment to the HDE. The supplement should be submitted to the Document Mail Center (HFZ-401), Office of Device Evaluation, Center for Devices and Radiological Health, Food and Drug Administration (FDA), 9200 Corporate Blvd., Rockville, Maryland 20850.

II. ADVERTISEMENTS

Advertisements and other descriptive printed materials issued by the HDE holder or private label distributor with respect to this device should not recommend or imply that the device may be used for any use that is not included in the FDA approved labeling for the device. If the FDA approval order has restricted the sale, distribution and use of the device to prescription use in accordance with 21 CFR 801.109 and specified that this restriction is being imposed in accordance with the provisions of section 520(e) of the Federal Food, Drug, and Cosmetic Act (the act) (21 U.S.C. 360j(e)) under the authority of section 515(d)(1)(B)(ii) of the act (21 U.S.C. 360e(d)(1)(B)(ii)), all advertisements and other descriptive printed material issued by the holder or distributor with respect to the device shall include a brief statement of the intended uses of the device and relevant warnings, precautions, side effects, and contraindications.

III. HDE SUPPLEMENTS

Before making any change affecting the safety or probable benefit of the device, the HDE holder should submit a supplement for review and approval by FDA unless a "Special HDE Supplement" is permitted as described under 21 CFR 814.39(d)(2) or an alternate submission is permitted as described under 21 CFR 814.39(e). All HDE supplements or alternate submissions must comply with the applicable requirements under 21 CFR 814.39 of the Premarket Approval (PMA) regulation and under 21 CFR 814.108 of the Humanitarian Device Exemption regulation. The review timeframe for HDE supplements is 75 days except for those submitted under 21 CFR 814.39(e).

Since all situations which require an HDE supplement cannot be briefly summarized, please consult the HDE regulation for further guidance. The guidance provided below is only for several key instances. In general, an HDE supplement must be submitted:

1) When unanticipated adverse effects, increases in the incidence of anticipated adverse effects, or device failures necessitate a labeling, manufacturing, or device modification; or

2) If the device is to be modified, and animal/laboratory or clinical testing is needed to determine if the modified device remains safe and continues to provide probable benefit.

HDE supplements submitted under 21 CFR 814.39(d)(2) "Special HDE Supplement - Changes Being Effected" are limited to the labeling, quality control, and manufacturing process changes as specified under this section of the regulation. This provision allows for the addition of, but not the replacement of previously approved, quality control specifications and test methods. These

changes may be implemented upon acknowledgment by FDA that the submission is being processed as a "Special HDE Supplement - Changes Being Effected." Please note that this acknowledgment is in addition to that issued by the Document Mail Center for all HDE supplements submitted. This procedure is not applicable to changes in device design, composition, specifications, circuitry, software, or energy source.

Alternate submissions permitted under 21 CFR 814.39(e) apply to changes that otherwise require approval of an HDE supplement before implementation and include the use of a *30-day HDE supplement* or *periodic postapproval report*. FDA must have previously indicated in an advisory opinion to the affected industry or in correspondence to the HDE holder that the alternate submission is permitted for the change. Before this can occur, FDA and the HDE holder must agree upon any needed testing, the testing protocol, the test results, the reporting format, the information to be reported, and the alternate submission to be used.

Please note that unlike the PMA process, a supplement may not be submitted for a new indication for use for a humanitarian use device (HUD). An HDE holder seeking a new indication for use for an HUD approved under the provisions of Subpart H of 21 CFR 814, must obtain a new designation of HUD status for the new indication for use and submit an original HDE application in accordance with §814.104. The application for the new indication for use may incorporate by reference any information or data previously submitted to the agency.

IV. POSTAPPROVAL RECORD KEEPING REQUIREMENTS

An HDE holder is required to maintain records of the names and addresses of the facilities to which the HUD has been shipped, correspondence with reviewing institutional review boards (IRBs), as well as any other information requested by a reviewing IRB or FDA.

V. POSTAPPROVAL REPORTING REQUIREMENTS

Continued approval of the HDE is contingent upon the submission of postapproval reports required under 21 CFR 814.84 and 21 CFR 814.126.

A. ANNUAL REPORT

Annual reports should be submitted at intervals of 1 year from the date of approval of the original HDE. Reports for supplements approved under the original HDE should be included in the next and subsequent periodic reports for the original HDE unless otherwise specified in the approval order for the HDE supplement. Three copies identified as "Annual Report" and bearing the applicable HDE reference number are to be submitted to the HDE Document Mail Center (HFZ-401), Center for Devices and Radiological Health, Food and Drug Administration, 9200 Corporate Blvd., Rockville, Maryland 20850. Reports should indicate the beginning and ending date of the period covered by the report and include the following information required by 21 CFR 814.126(b)(1):

1. An update of the information required under §814.102(a) in a separately bound volume;

2. An update of the information required under §814.104(b)(2), (b)(3), and (b)(5);

3. The number of devices that have been shipped or sold and, if the number shipped or sold exceeds 4,000, an explanation and estimate of the number of devices used per patient. If a single device is used on multiple patients, an estimate of the number of patients treated or diagnosed using the device together with an explanation of the basis for the estimate;

4. Information describing the applicant's clinical experience with the device. This shall include safety information that is known or reasonably should be known to the applicant, a summary of medical device reports made pursuant to 21 CFR 803, any data generated from postmarketing studies, and information (whether published or unpublished) that is known or reasonably expected to be known by the applicant that may affect an evaluation of the safety of the device or that may affect the statement of contraindications, warnings, precautions, and adverse reactions in the device labeling; and

5. A summary of any changes made to the device in accordance with supplements submitted under §814.108 and any changes required to be reported to FDA under §814.39(b).

B. **ADVERSE REACTION AND DEVICE DEFECT REPORTING**

As provided by 21 CFR 814.82(a)(9), FDA has determined that in order to provide continued reasonable assurance of the safety and probable benefit of the device, the holder shall submit three copies of a written report identified, as applicable, as an "Adverse Reaction Report" or "Device Defect Report" to the Document Mail Center (HFZ-401), Office of Device Evaluation, Center for Devices and Radiological Health, Food and Drug Administration, 9200 Corporate Blvd., Rockville, Maryland 20850. Such reports should be submitted within 10 days after the HDE holder receives or has knowledge of information concerning:

(1) A mixup of the device or its labeling with another article.

(2) Any adverse reaction, side effect, injury, toxicity, or sensitivity reaction that is attributable to the device and

(a) has not been addressed by the device's labeling or

(b) has been addressed by the device's labeling, but is occurring with unexpected severity or frequency.

(3) Any significant chemical, physical or other change or deterioration in the device or any failure of the device to meet the specifications established in the approved HDE that could not cause or contribute to death or serious injury but are not correctable by adjustments or other maintenance procedures described in the approved labeling. The report shall include a discussion of the HDE holder's assessment of the change, deterioration or failure and any proposed or implemented corrective action by the firm. When such events are correctable by adjustments or other maintenance procedures described in the approved labeling, all such events known to the holder shall be included in the "Annual Report" described under "Postapproval Reports" above unless

otherwise specified in the conditions of approval for this HDE. This postapproval report shall appropriately categorize these events and include the number of reported and otherwise known instances of occurrence for each category during the reporting period. Additional information regarding the events discussed above shall be submitted by the HDE holder when determined by FDA to be necessary to provide continued reasonable assurance of the safety and probable benefit of the device for its intended use.

C. **REPORTING UNDER THE MEDICAL DEVICE REPORTING REGULATION**

The Medical Device Reporting regulation (MDR) (21 CFR 803) became effective on July 31, 1996 and requires that all manufacturers and importers of medical devices, including in vitro diagnostic devices, report to FDA whenever they receive or otherwise became aware of information that reasonably suggests that one of its marketed devices:

(1) may have caused or contributed to a death or serious injury; or

(2) has malfunctioned and that the device or a similar device marketed by the manufacturer or importer would be likely to cause or contribute to a death or serious injury if the malfunction were to recur.

Events subject to reporting under the MDR regulation may also be subject to the above "Adverse Reaction and Device Defect Reporting" requirements. FDA has determined, however, that such duplicative reporting is unnecessary. Therefore, whenever an event involving a device is subject to reporting under both the MDR regulation and the "Adverse Reaction and Device Defect Reporting" requirements, the report should be submitted in compliance with Part 803 and identified with the HDE reference number to Food and Drug Administration, Center for Devices and Radiological Health, Medical Device Reporting, PO Box 3002, Rockville, Maryland 20847-3002. If you have MDR regulation questions, please send an e-mail to RSMB@CDRH.FDA.GOV or call (301) 594-2735.

Events included in periodic reports to the HDE that have also been reported under the MDR regulation must be so identified in the periodic report to the HDE to prevent duplicative entry into FDA information systems.

Copies of the MDR regulation and FDA publications, entitled "An Overview of the Medical Device Reporting Regulation" and "Medical Device Reporting for Manufacturers," are available on the CDRH WWW Home Page (http://www.fda.gov/cdrh), through CDRH's Fact-on-Demand (FOD) at 800-899-0381 (FOD # 336, 1336, 509 and 987) or by written request to the address below or by telephoning 1-800-638-2041.

Division of Small Manufacturers International
and Consumer Assistance (HFZ-220)
Center for Devices and Radiological Health
Food and Drug Administration

7

1350 Piccard Drive
Rockville, Maryland 20850

US005258494A

United States Patent [19]

Oppermann et al.

[11] Patent Number: 5,258,494

[45] Date of Patent: Nov. 2, 1993

[54] OSTEOGENIC PROTEINS

[75] Inventors: **Hermann Oppermann; Thangavel Kuberasampath**, both of Medway; **David C. Rueger**, West Roxbury; **Engin Ozkaynak**, Milford, all of Mass.

[73] Assignee: **Stryker Corporation**, Kalamazoo, Mich.

[21] Appl. No.: **995,345**

[22] Filed: **Dec. 22, 1992**

Related U.S. Application Data

[60] Continuation of Ser. No. 621,988, Dec. 4, 1990, abandoned, which is a division of Ser. No. 315,342, Feb. 23, 1989, Pat. No. 5,011,691, which is a continuation-in-part of Ser. No. 232,630, Aug. 15, 1988, abandoned, which is a continuation-in-part of Ser. No. 179,406, Apr. 8, 1988, Pat. No. 4,968,590.

[51] Int. Cl.[5] C07K 5/00; C07K 7/00; C07K 15/00; A61K 37/02

[52] U.S. Cl. **530/326**; 530/327; 530/328; 530/395; 530/840; 435/69.1; 435/172.3

[58] Field of Search 435/69.1, 91, 172.3, 435/235.1, 240.2, 252, 255, 320.1; 536/27; 530/359, 326, 327, 328, 395, 840, 300, 350; 935/18, 22, 41, 55, 60, 69, 70, 72, 84

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8505274	12/1985	PCT Int'l Appl. .
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Primary Examiner—Nathan M. Nutter
Attorney, Agent, or Firm—Testa, Hurwitz & Thibeault

[57] **ABSTRACT**

Disclosed are 1) osteogenic devices comprising a matrix containing osteogenic protein and methods of inducing endochondral bone growth in mammals using the devices; 2) amino acid sequence data, amino acid composition, solubility properties, structural features, homologies and various other data characterizing osteogenic proteins, 3) methods of producing osteogenic proteins using recombinant DNA technology, and 4) osteogenically and chondrogenically active synthetic protein constructs.

17 Claims, 30 Drawing Sheets

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FIG. 1A-1

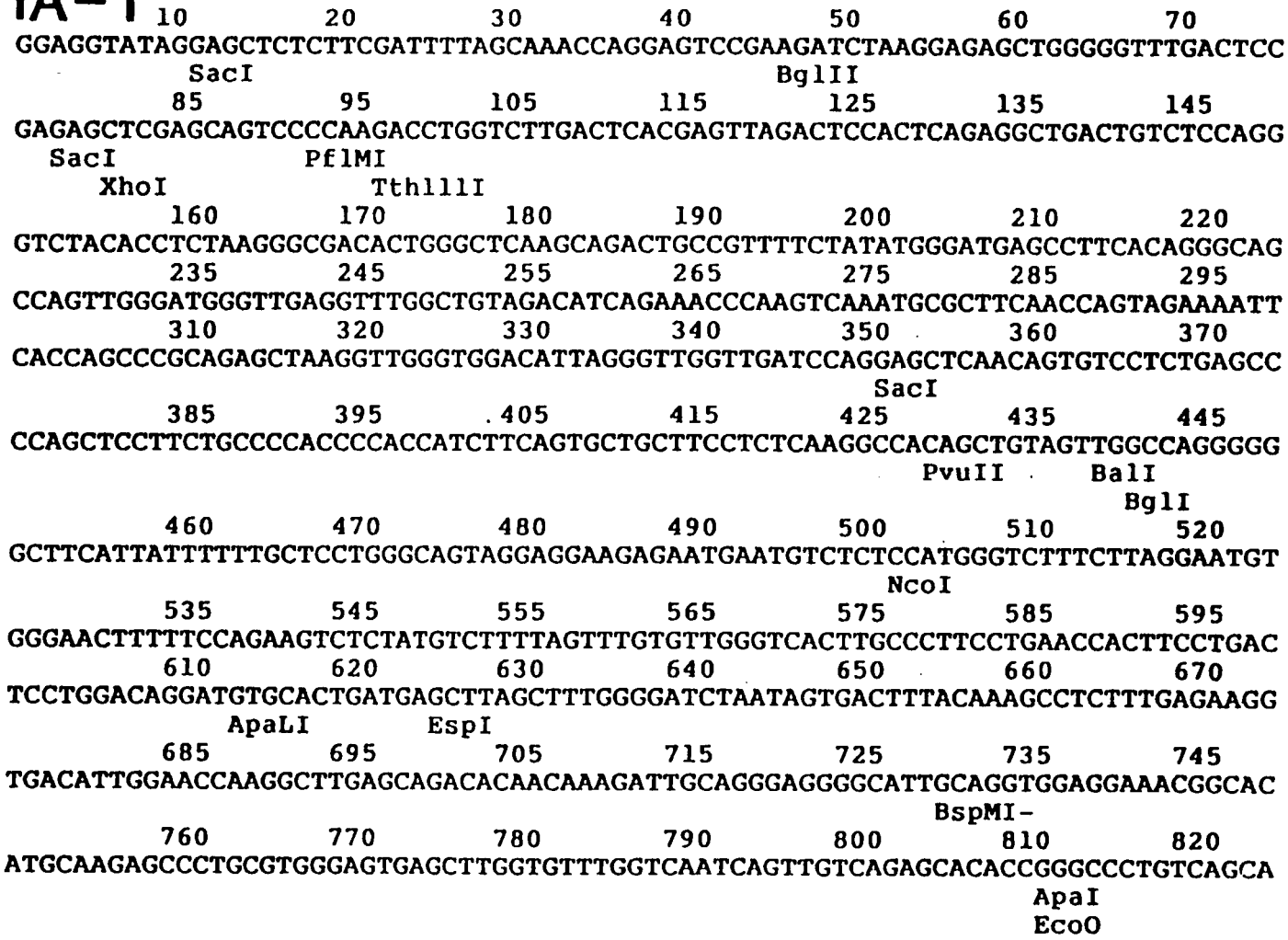
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FIG. 1A-2

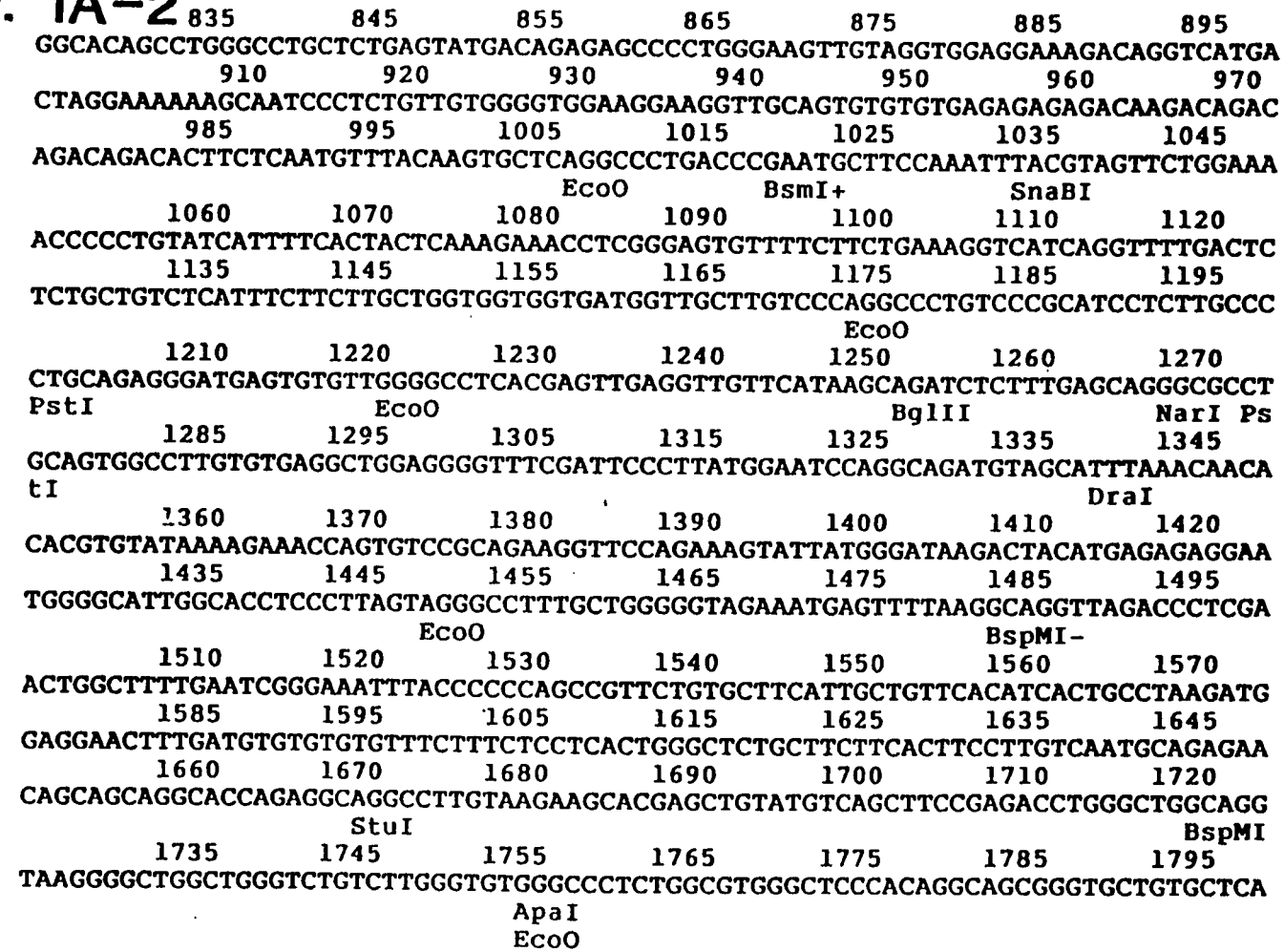
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FIG. 1A-3

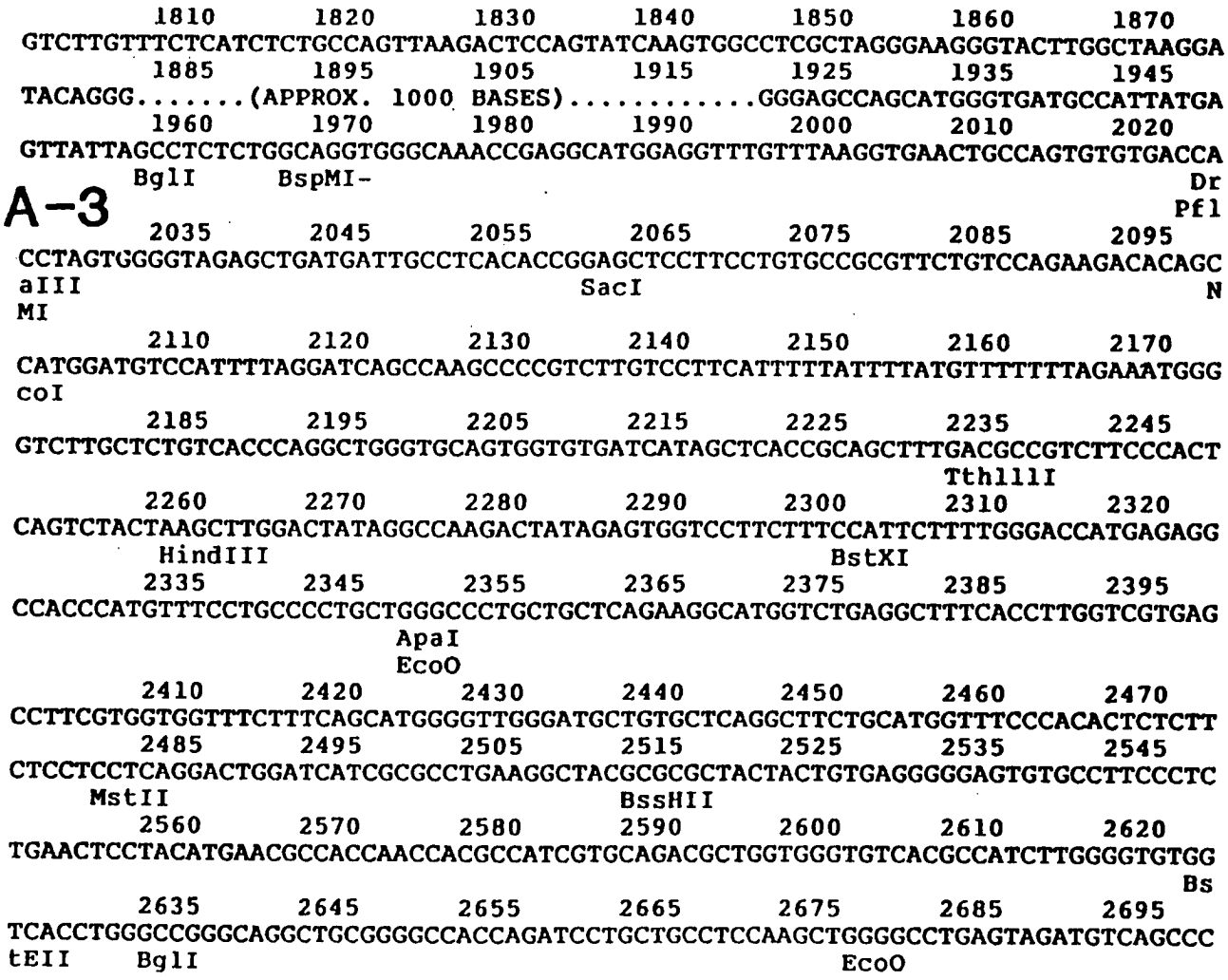
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FIG. 1A-4

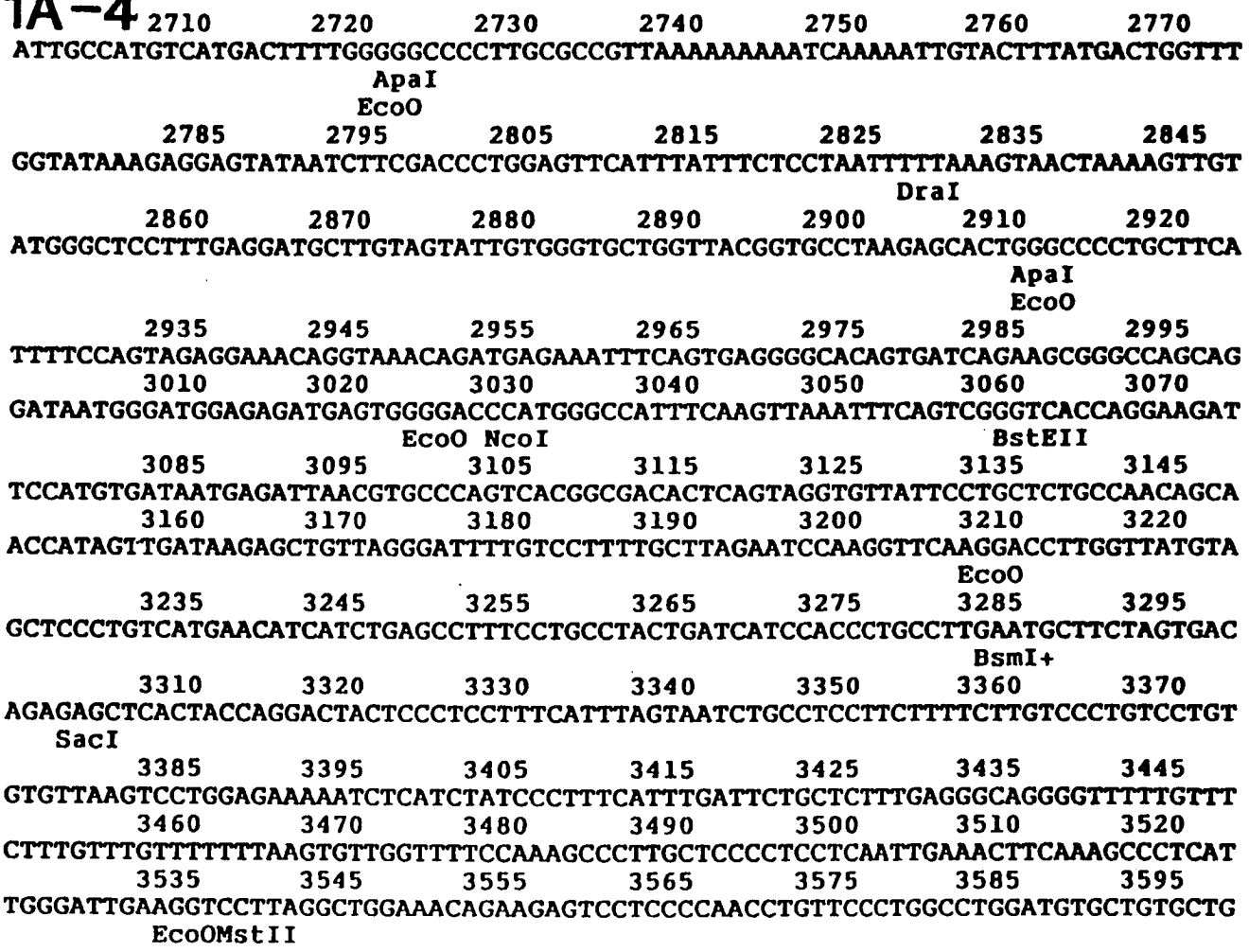
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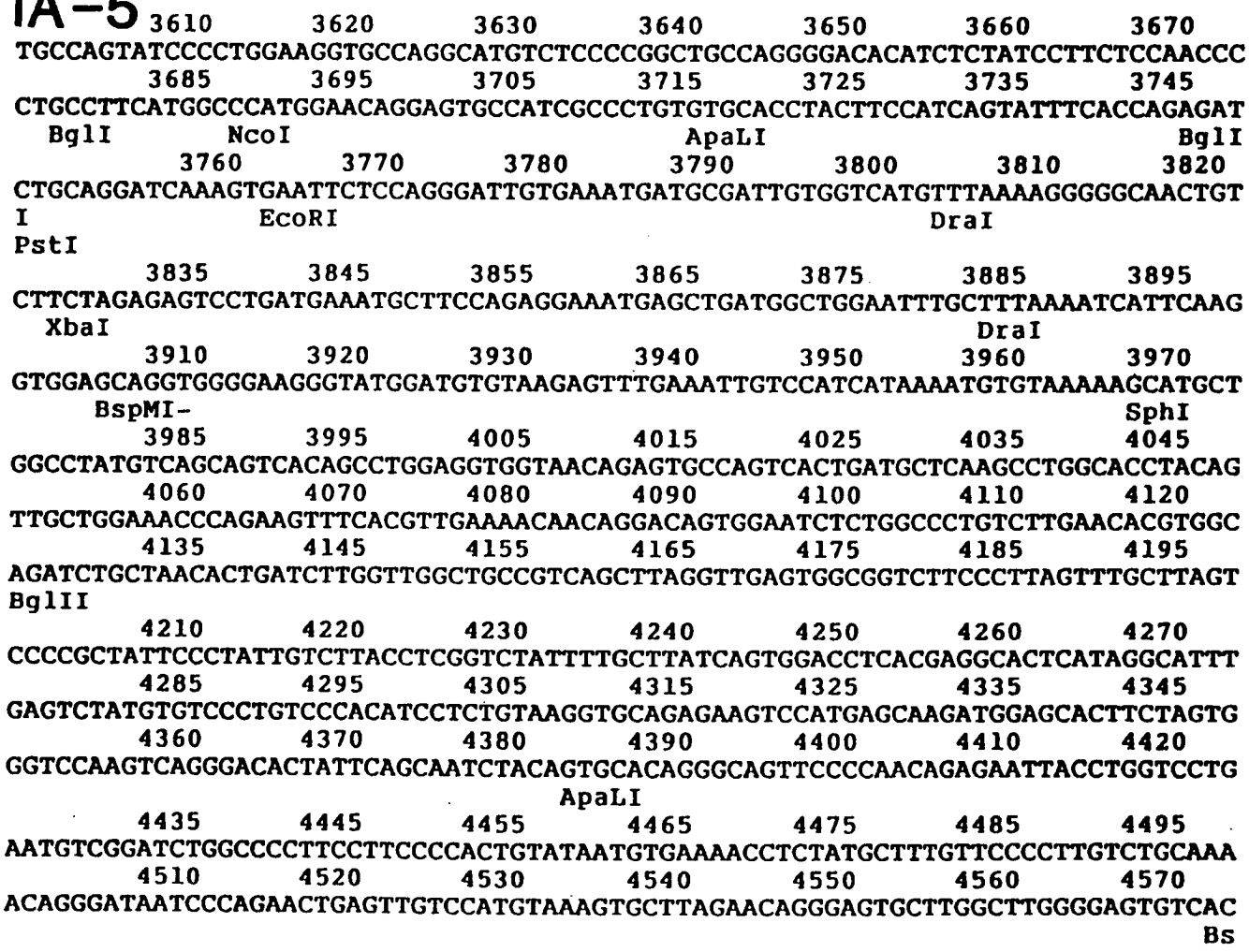
FIG. 1A-5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3760 3770 3780 3790 3800 3810 3820
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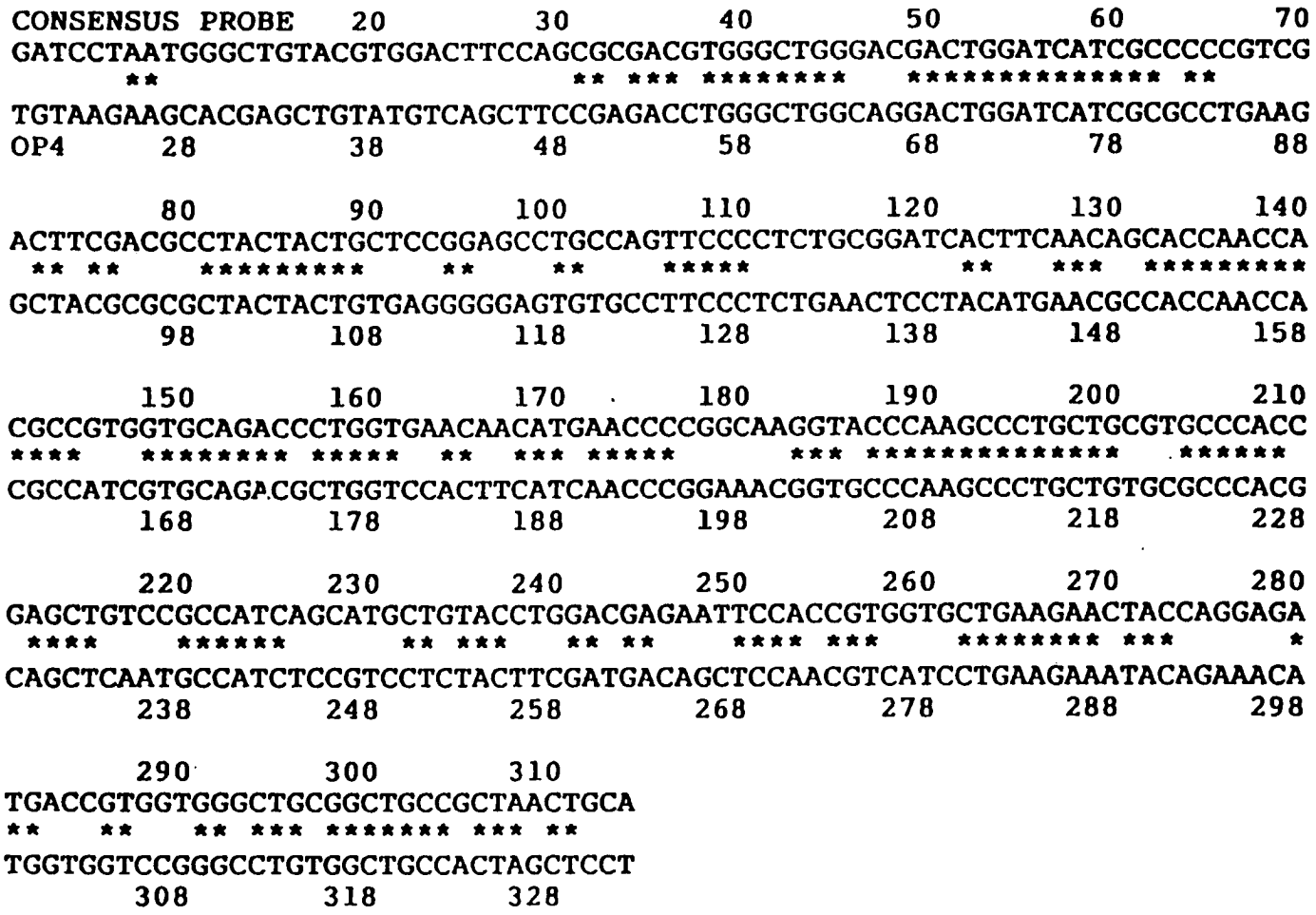
I EcoRI DraI
PstI
3835 3845 3855 3865 3875 3885 3895
CTTCTAGAGAGTCCTGATGAAATGCTTCCAGAGGAAATGAGCTGATGGCTGGAATTTGCTTTAAAATCATTCAAG
XbaI DraI
3910 3920 3930 3940 3950 3960 3970
GTGGAGCAGGTGGGGAAGGGTATGGATGTGTAAGAGTTTGAAATTGTCCATCATAAAATGTGTAAAAAGCATGCT
BspMI- SphI
3985 3995 4005 4015 4025 4035 4045
GGCCTATGTCAGCAGTCACAGCCTGGAGGTGGTAACAGAGTGCCAGTCACTGATGCTCAAGCCTGGCACCTACAG
4060 4070 4080 4090 4100 4110 4120
TTGCTGGAAACCCAGAAGTTTCACGTTGAAAACAACAGGACAGTGGAATCTCTGGCCCTGTCTTGAACACGTGGC
4135 4145 4155 4165 4175 4185 4195
AGATCTGCTAACACTGATCTTGGTTGGCTGCCGTCAGCTTAGGTTGAGTGGCGGTCTTCCCTTAGTTTGCTTAGT
BglII
4210 4220 4230 4240 4250 4260 4270
CCCCGCTATTCCCTATTGTCTTACCTCGGTCTATTTTGCTTATCAGTGGACCTCACGAGGCACTCATAGGCATTT
4285 4295 4305 4315 4325 4335 4345
GAGTCTATGTGTCCCTGTCCCACATCCTCTGTAAGGTGCAGAGAAGTCCATGAGCAAGATGGAGCACTTCTAGTG
4360 4370 4380 4390 4400 4410 4420
GGTCCAAGTCAGGGACACTATTCAGCAATCTACAGTGCACAGGGCAGTTCCCCAACAGAGAATTACCTGGTCCTG
ApaLI
4435 4445 4455 4465 4475 4485 4495
AATGTCGGATCTGGCCCCTTCCTTCCCCACTGTATAATGTGAAAACCTCTATGCTTTGTTCCCCTTGTCTGCAAA
4510 4520 4530 4540 4550 4560 4570
ACAGGGATAATCCCAGAACTGAGTTGTCCATGTAAAGTGCTTAGAACAGGGAGTGCTTGGCTTGGGGAGTGTCAC
Bs

FIG. 1A-6

```
        4585       4595       4605       4615       4625       4635       4645
CTGCAGTCATTCATTATGCCCAGACAGGATGTTTCTTTATAGAAACGTGGAGGCCAGTTAGAACGACTCACCGCT
pMI+
PstI
        4660       4670       4680       4690       4700       4710       4720
TCTCACCACTGCCCATGTTTTGGTGTGTGTTTCAGGTCCACTTCATCAACCCGGAAACGGTGCCCAAGCCCTGCT
             PflMI
        4735       4745       4755       4765       4775       4785       4795
GTGCGCCCACGCAGCTCAATGCCATCTCCGTCCTCTACTTCGATGACAGCTCCAACGTCATCCTGAAGAAATACA
        4810       4820       4830       4840
GAAACATGGTGGTCCGGGCCTGTGGCTGCCACTAGCTCCTCCGA
```

FIG. 1B


CONSENSUS PROBE 20 30 40 50 60 70
GATCCTAATGGGCTGTACGTGGACTTCCAGCGCGACGTGGGCTGGGACGACTGGATCATCGCCCCCGTCG
TGTAAGAAGCACGAGCTGTATGTCAGCTTCCGAGACCTGGGCTGGCAGGACTGGATCATCGCGCCTGAAG
OP4 28 38 48 58 68 78 88
80 90 100 110 120 130 140
ACTTCGACGCCTACTACTGCTCCGGAGCCTGCCAGTTCCCCTCTGCGGATCACTTCAACAGCACCAACCA
GCTACGCGCGCTACTACTGTGAGGGGGAGTGTGCCTTCCCTCTGAACTCCTACATGAACGCCACCAACCA
98 108 118 128 138 148 158
150 160 170 180 190 200 210
CGCCGTGGTGCAGACCCTGGTGAACAACATGAACCCCGGCAAGGTACCCAAGCCCTGCTGCGTGCCCACC
CGCCATCGTGCAGACGCTGGTCCACTTCATCAACCCGGAAACGGTGCCCAAGCCCTGCTGTGCGCCCACG
168 178 188 198 208 218 228
220 230 240 250 260 270 280
GAGCTGTCCGCCATCAGCATGCTGTACCTGGACGAGAATTCCACCGTGGTGCTGAAGAACTACCAGGAGA
CAGCTCAATGCCATCTCCGTCCTCTACTTCGATGACAGCTCCAACGTCATCCTGAAGAAATACAGAAACA
238 248 258 268 278 288 298
290 300 310
TGACCGTGGTGGGCTGCGGCTGCCGCTAACTGCA
TGGTGGTCCGGGCCTGTGGCTGCCACTAGCTCCT
308 318 328

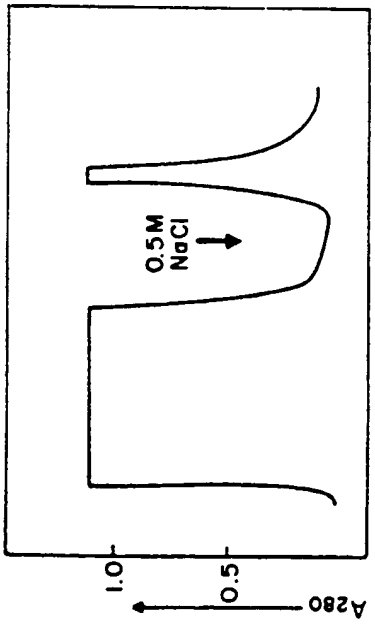


FIG. 2A

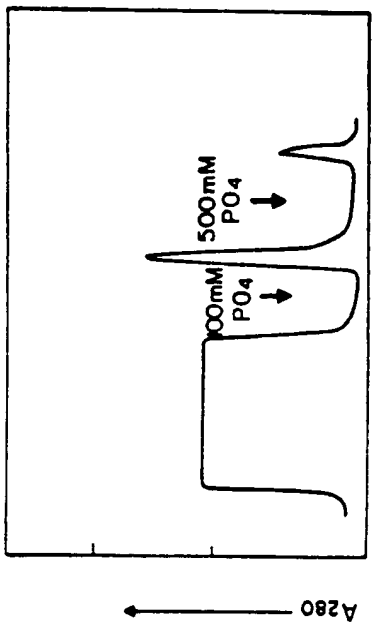


FIG. 2B

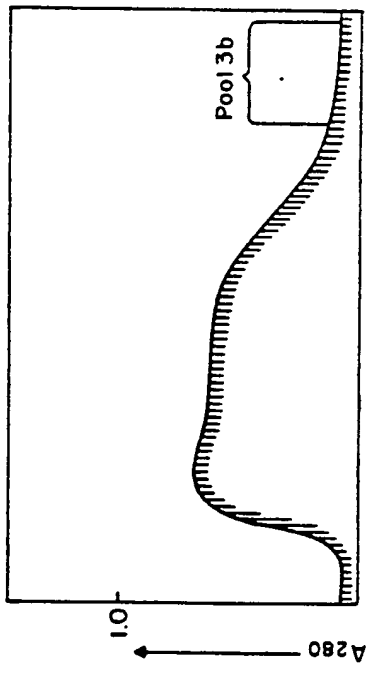


FIG. 2C

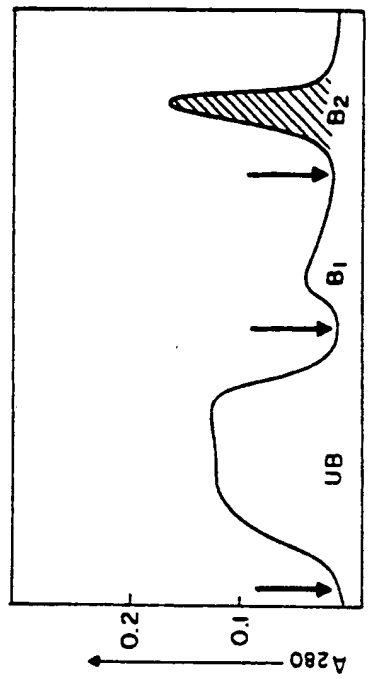


FIG. 2D

Fig. 3A

Fig. 3B

Fig. 4A

Fig. 4B

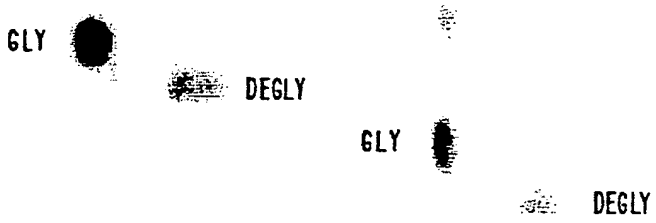


Fig. 5A Fig. 5B

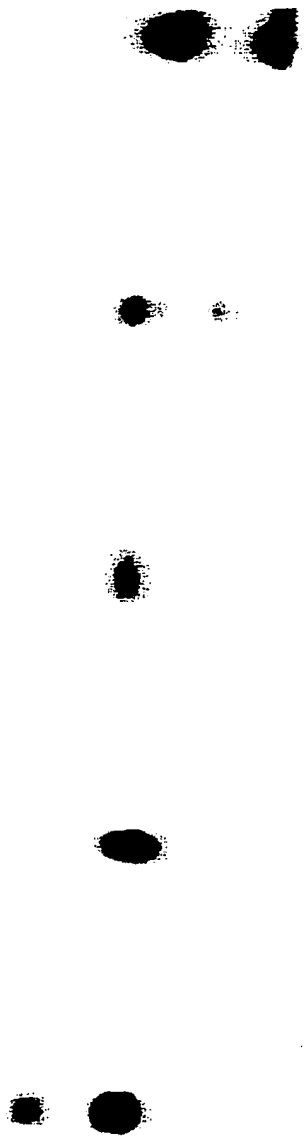


Fig. 6E

Fig. 6D

Fig. 6C

Fig. 6B

Fig. 6A

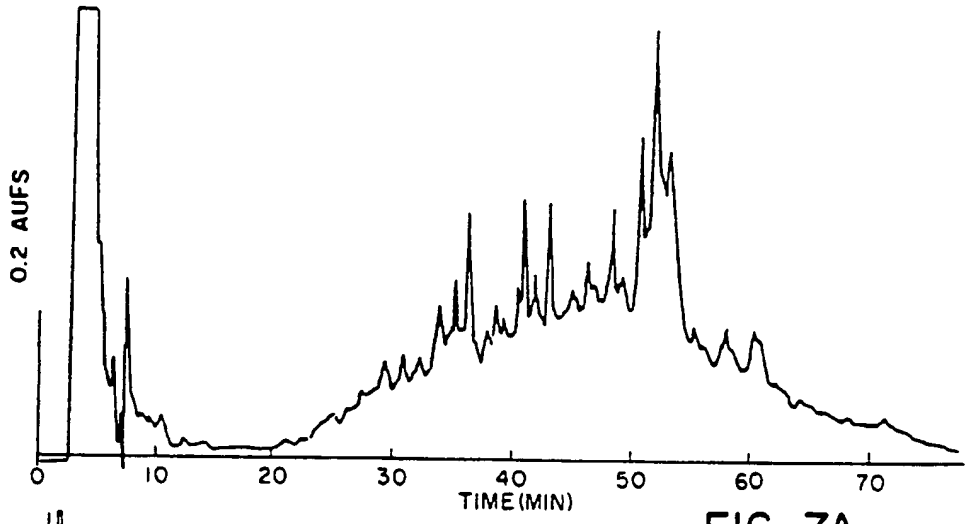


FIG. 7A

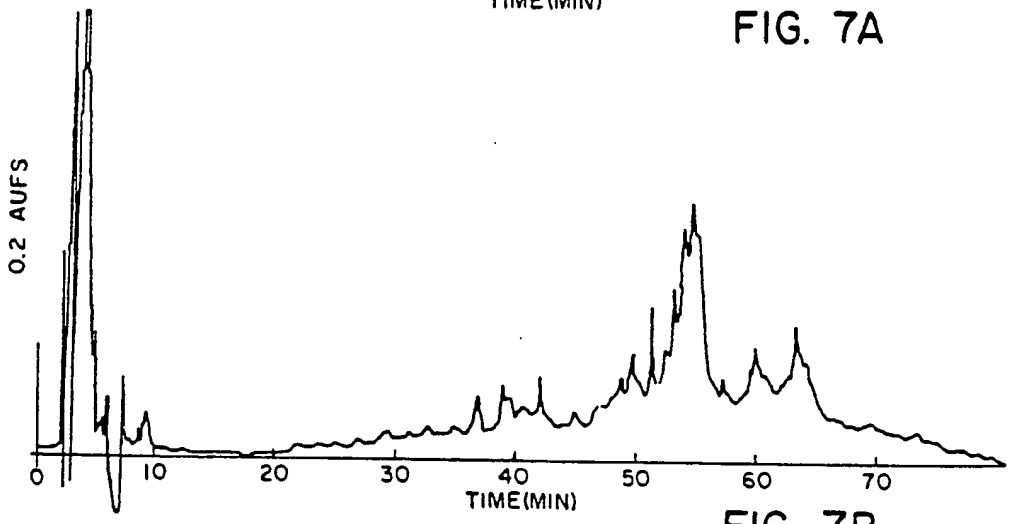


FIG. 7B

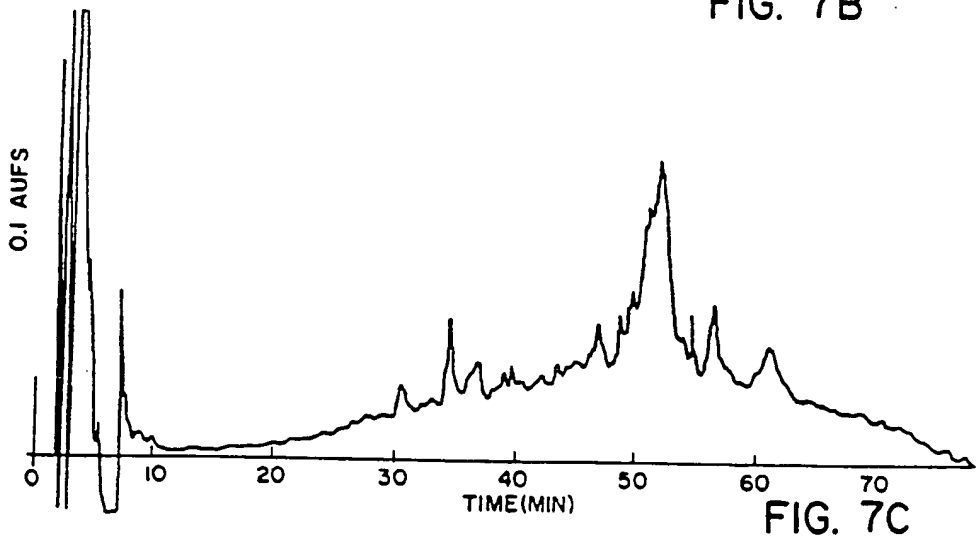


FIG. 7C

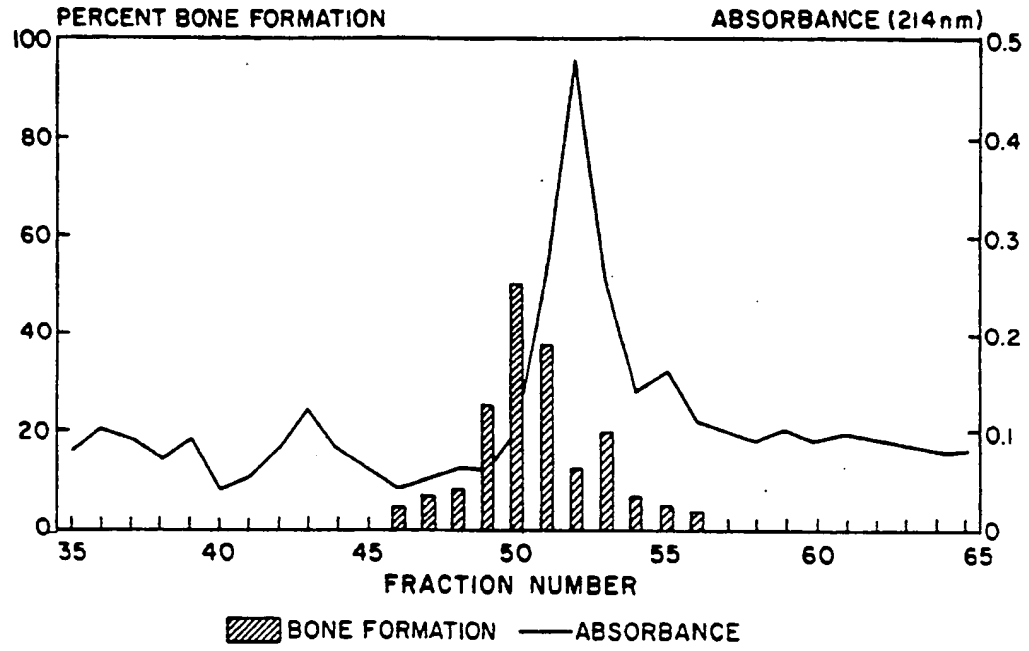

PERCENT BONE FORMATION
ABSORBANCE (214nm)
100
80
60
40
20
0
0.5
0.4
0.3
0.2
0.1
0
35
40
45
50
55
60
65
FRACTION NUMBER
BONE FORMATION
ABSORBANCE

FIG. 8

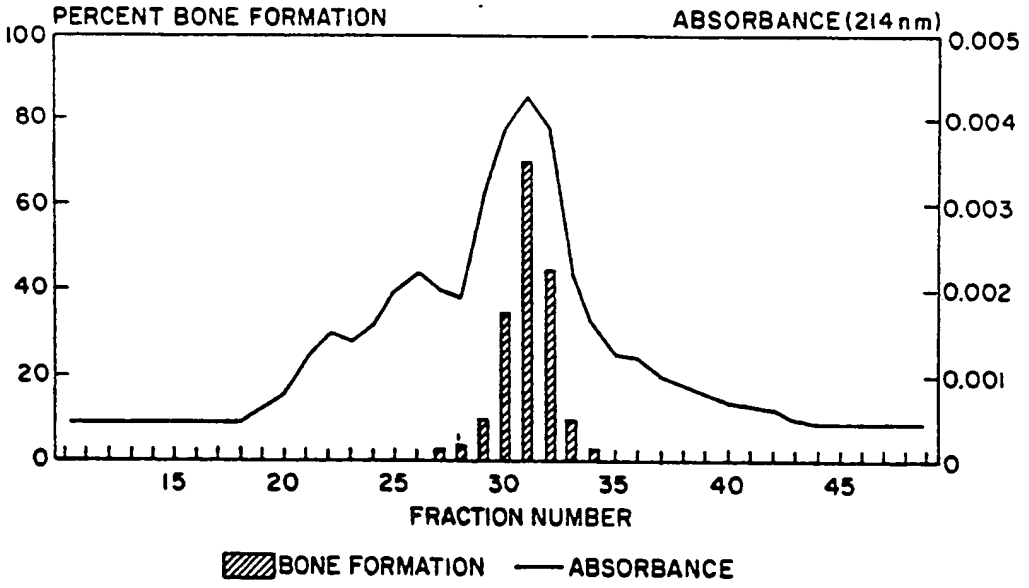

PERCENT BONE FORMATION
ABSORBANCE (214nm)
100
80
60
40
20
0
0.005
0.004
0.003
0.002
0.001
0
15
20
25
30
35
40
45
FRACTION NUMBER
BONE FORMATION
ABSORBANCE

FIG. 9

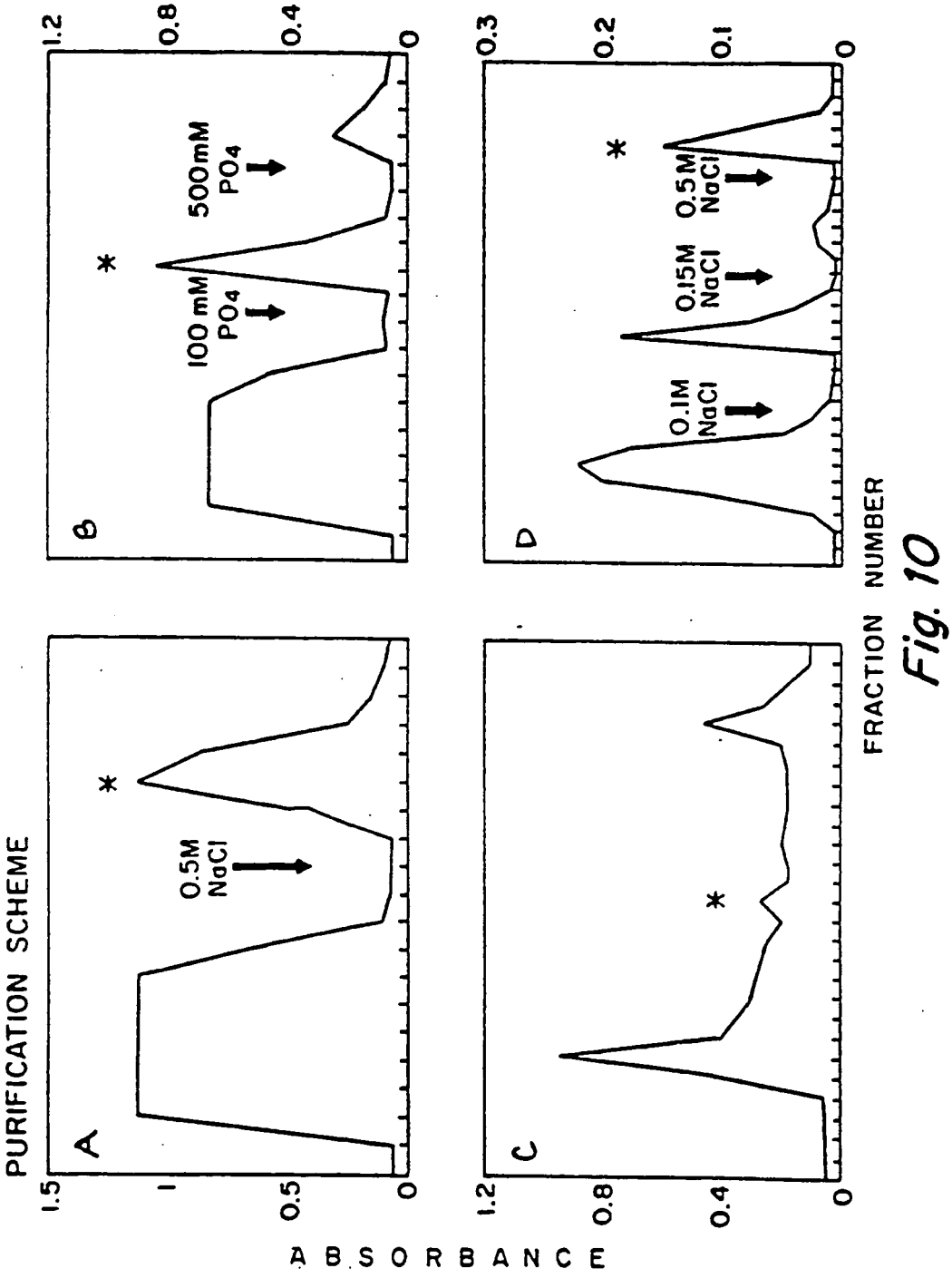

PURIFICATION SCHEME
A
0.5M NaCl
*
1.5
1
0.5
0
B
100 mM PO4
500mM PO4
*
1.2
0.8
0.4
0
C
*
1.2
0.8
0.4
0
D
0.1M NaCl
0.15M NaCl
0.5M NaCl
*
0.3
0.2
0.1
0
ABSORBANCE
FRACTION NUMBER

Fig. 10

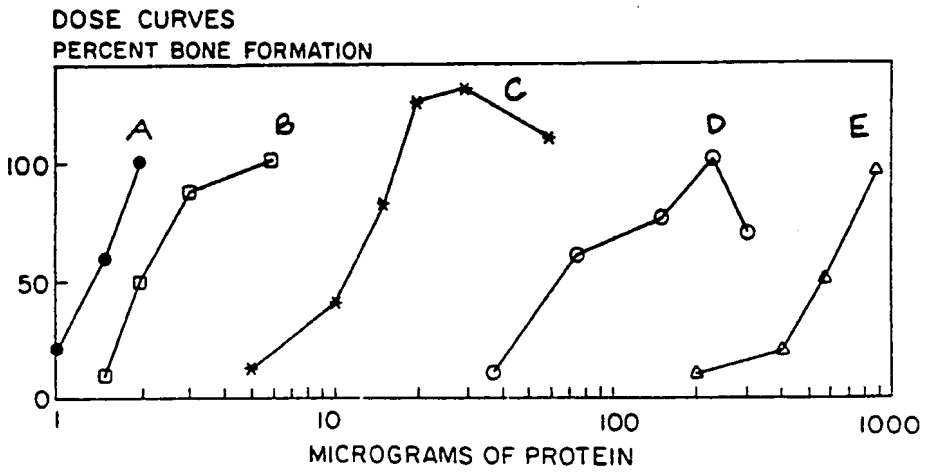

DOSE CURVES
PERCENT BONE FORMATION
A
B
C
D
E
100
50
0
1
10
100
1000
MICROGRAMS OF PROTEIN

FIG. 11

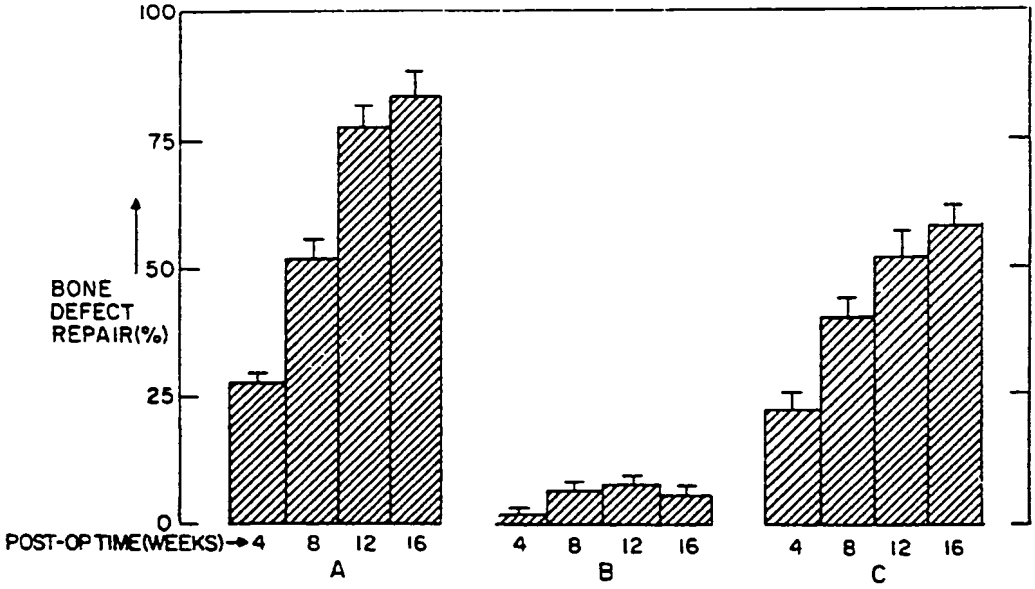

100
75
50
25
0
BONE
DEFECT
REPAIR(%)
POST-OP TIME(WEEKS)→4 8 12 16
A
4 8 12 16
B
4 8 12 16
C

FIG. 12

FIG. 13

```
        10         20         30         40         50
GATCCTAATGGGCTGTACGTGGACTTCCAGCGCGACGTGGGCTGGGACGA
 D  P  N  G  L  Y  V  D  F  Q  R  D  V  G  W  D  D

        60         70         80         90        100
CTGGATCATCGCCCCCGTCGACTTCGACGCCTACTACTGCTCCGGAGCCT
  W  I  I  A  P  V  D  F  D  A  Y  Y  C  S  G  A

       110        120        130        140        150
GCCAGTTCCCCTCTGCGGATCACTTCAACAGCACCAACCACGCCGTGGTG
C  Q  F  P  S  A  D  H  F  N  S  T  N  H  A  V  V

       160        170        180        190        200
CAGACCCTGGTGAACAACATGAACCCCGGCAAGGTACCCAAGCCCTGCTG
 Q  T  L  V  N  N  M  N  P  G  K  V  P  K  P  C  C

       210        220        230        240        250
CGTGCCCACCGAGCTGTCCGCCATCAGCATGCTGTACCTGGACGAGAATT
  V  P  T  E  L  S  A  I  S  M  L  Y  L  D  E  N

       260        270        280        290        300
CCACCGTGGTGCTGAAGAACTACCAGGAGATGACCGTGGTGGGCTGCGGC
S  T  V  V  L  K  N  Y  Q  E  M  T  V  V  G  C  G

       310
TGCCGCTAACTGCAG
 C  R  *
```

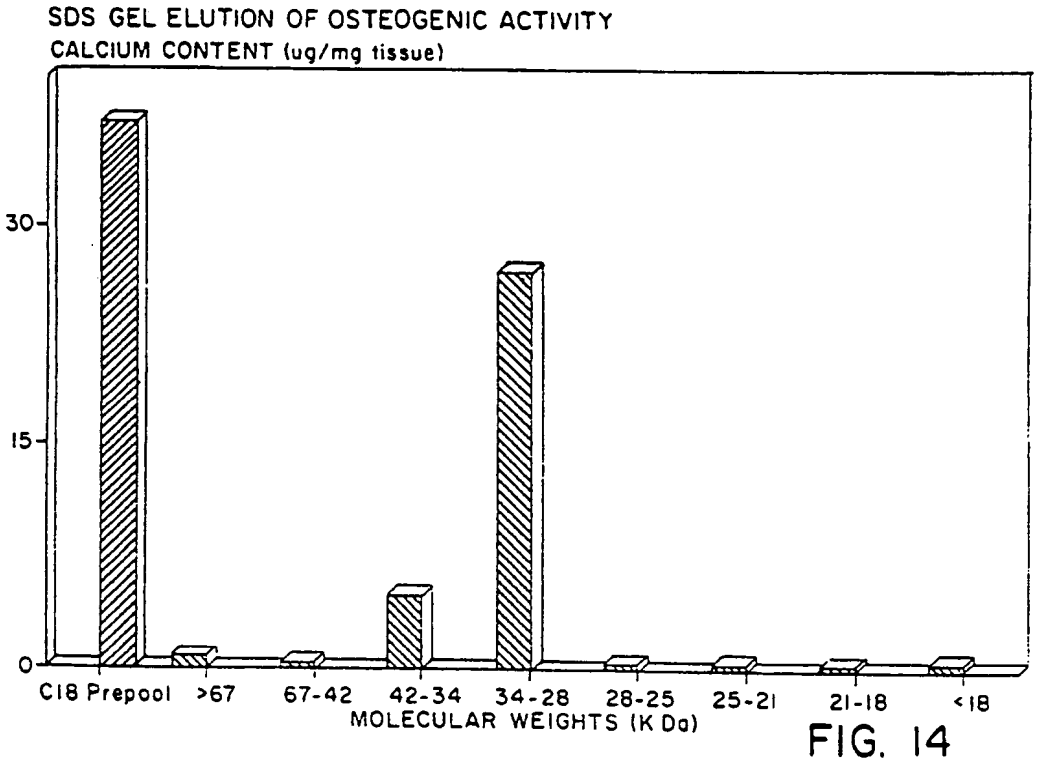

SDS GEL ELUTION OF OSTEOGENIC ACTIVITY
CALCIUM CONTENT (ug/mg tissue)
30
15
0
C18 Prepool
>67
67-42
42-34
34-28
28-25
25-21
21-18
<18
MOLECULAR WEIGHTS (K Da)

FIG. 14

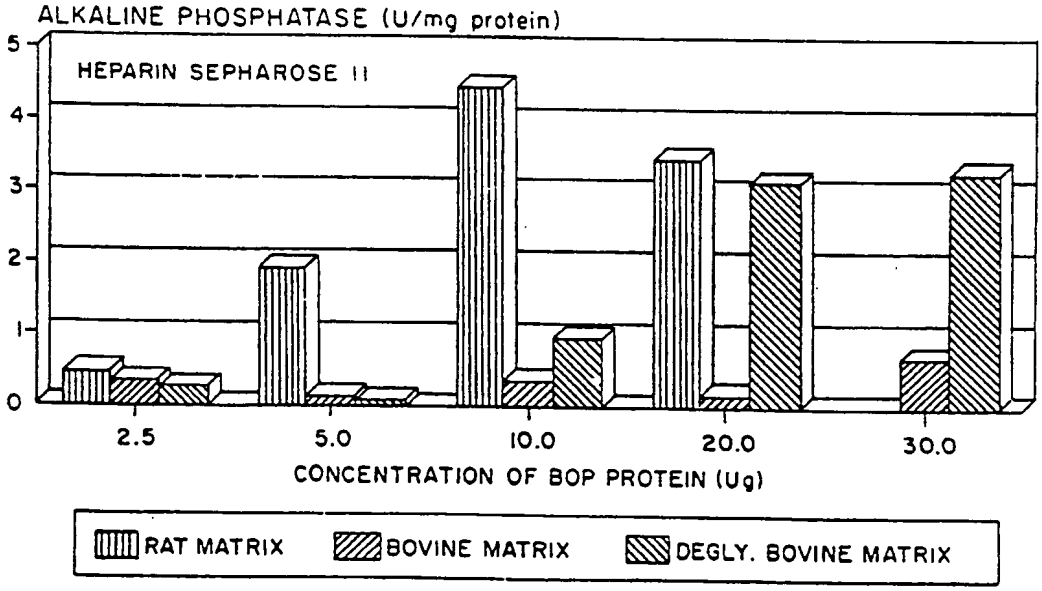

ALKALINE PHOSPHATASE (U/mg protein)
HEPARIN SEPHAROSE II
5
4
3
2
1
0
2.5
5.0
10.0
20.0
30.0
CONCENTRATION OF BOP PROTEIN (Ug)
RAT MATRIX
BOVINE MATRIX
DEGLY. BOVINE MATRIX

FIG. 19

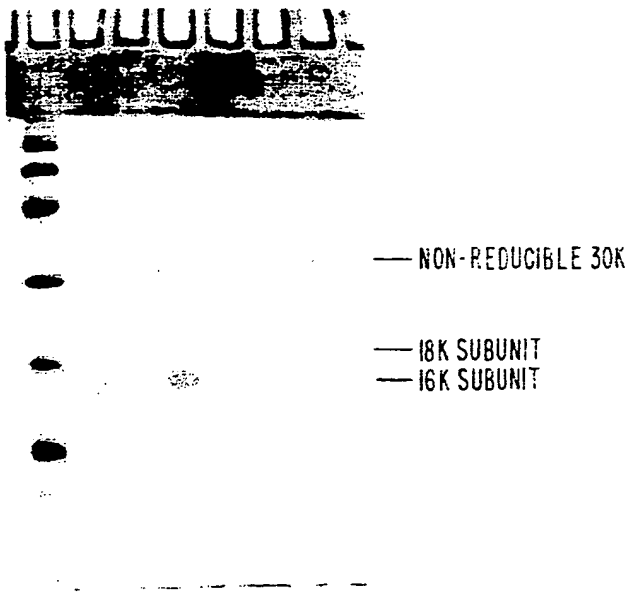


Fig. 15

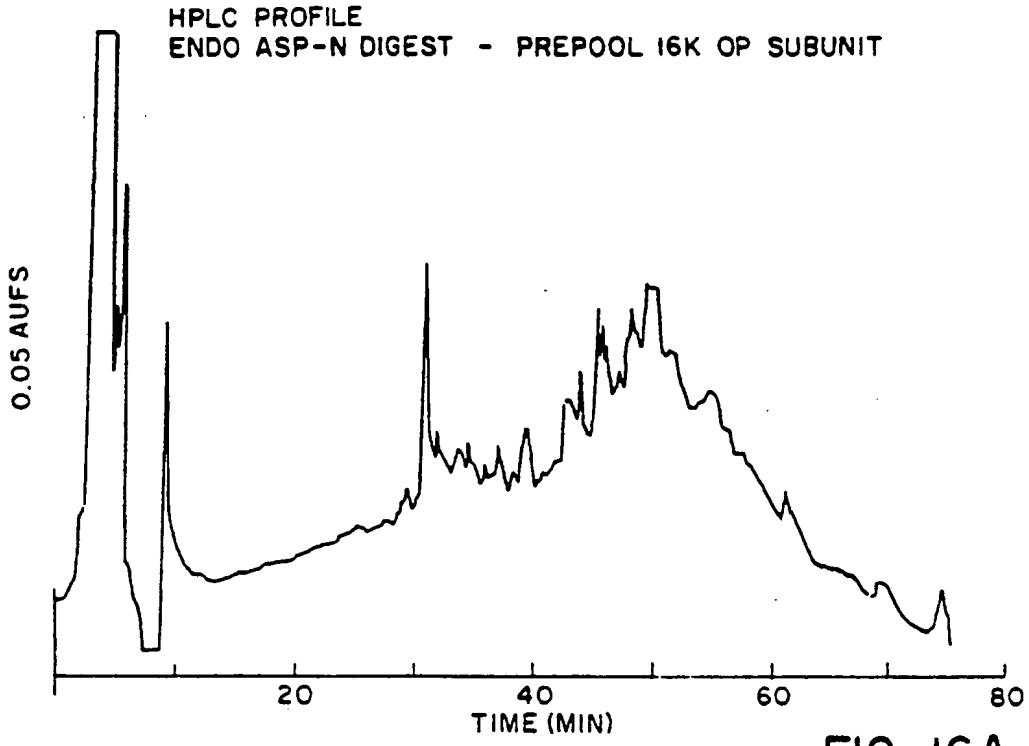


FIG. 16A

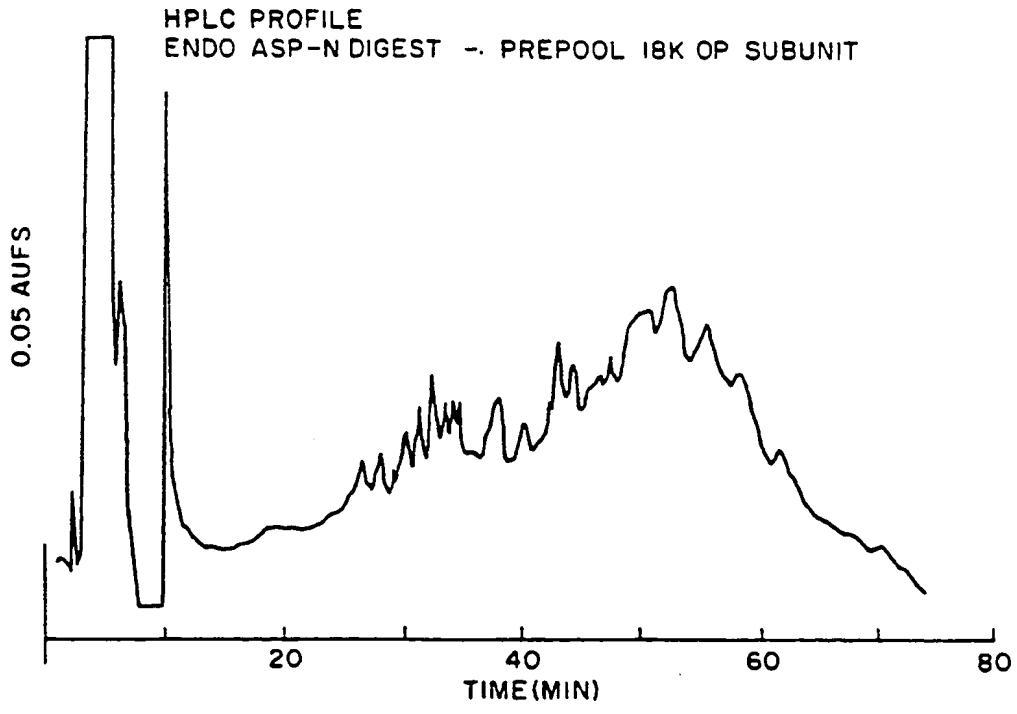


FIG. 16B

Fig. 17A

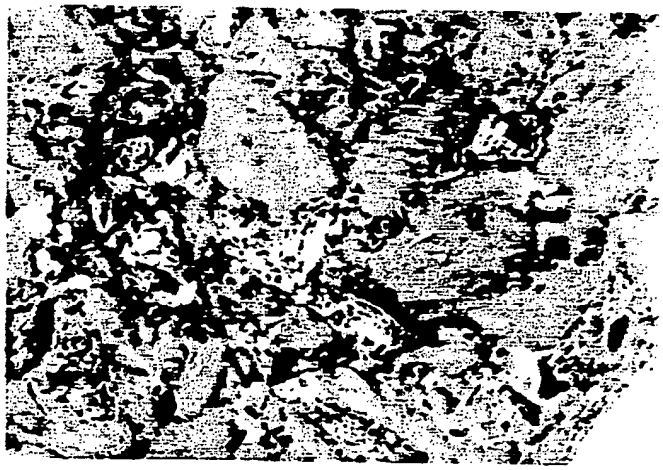


Fig. 17B

Fig. 17C

COP1 COP3 COP4 COP5 COP7	VG1	DPP	OP1	CBMP2 a)	CBMP2 b)	CBMP3 (bov)	beta-Inhibin a)	beta-Inhibin b)	TGF-beta 1	TGF-beta 2	TGF-beta 3	MIS	alpha Inhib	consensus choices
	C	C	C	C		C	C	C	C			C	C	c
	K	R	K	K	R	A	C	C	C			A	H	k,r
<P P P P P>	K	R	K	R		R	K	R	V	L	V	L	R	k,r
<N N N N N>	R	H	H	H		R	K	Q	R			R	V	h,k,r,q
<G G G G G>	H	S	E	P	S	Y	Q	Q	Q	P	P	E	A	p,s,e,q
L L L L L	L	L	L	L		L	F	F	L			L	L	l
Y Y Y Y Y	Y	Y	Y	Y		K	F	F	Y			S	N	y,f
V V V V V	V	V	V	V		V	V	I	I			V	I	v,i
D D D D D	E	D	S	D		D	S	D	D			D	S	d,e,s
F F F F F	F	F	F	F		F	F	F	F			L	F	f
Q Q S S S	K	S	R	S		A	K	R	R	K	R	R	Q	k,r,s
R R									K	R	Q	A		
D D D D D	D	D	D	D		D	D		D			E	E	d
V V V V V	V	V	L	V		I	I	I	L			R	L	v,l,i
G G G G G	G	G	G	G		G	G	G	G			S	G	g
W W W W W	W	W	W	W		W	W	W	W				W	w
D D D D N	Q	D	Q	N		S	N	N	K				E	q,n,d,e,s
D D D D D	N	D	D	D		E	D	D	-				R	d,e,n
W W W W W	W	W	W	W		W	W	W	W				W	w
I I I I I	V	I	I	I		I	I	I	I	I	V	V	I	i,v
I V V V V	I	V	I	V		I	I	I	H			L	V	i,v
A A A A A	A	A	A	A		S	A	A	E			I	Y	a,s
P P P P P	P	P	P	P		P	P	P	P			P	P	p
V P P P P	Q	L	E	P		K	S	T	K			E	P	e,q,p*
D G G G G	G	G	G	G		S	G	G	G			T	S	g,s
F Y Y Y Y	Y	Y	Y	Y		F	Y	Y	Y			Y	F	y,f
D Q Q Q H	M	D	A	H		D	H	Y	H	N	Y	Q	I	h,d,n,q,y*
A A A A A	A	A	A	A		A	A	G	A			A	F	a

FIG. 18-1

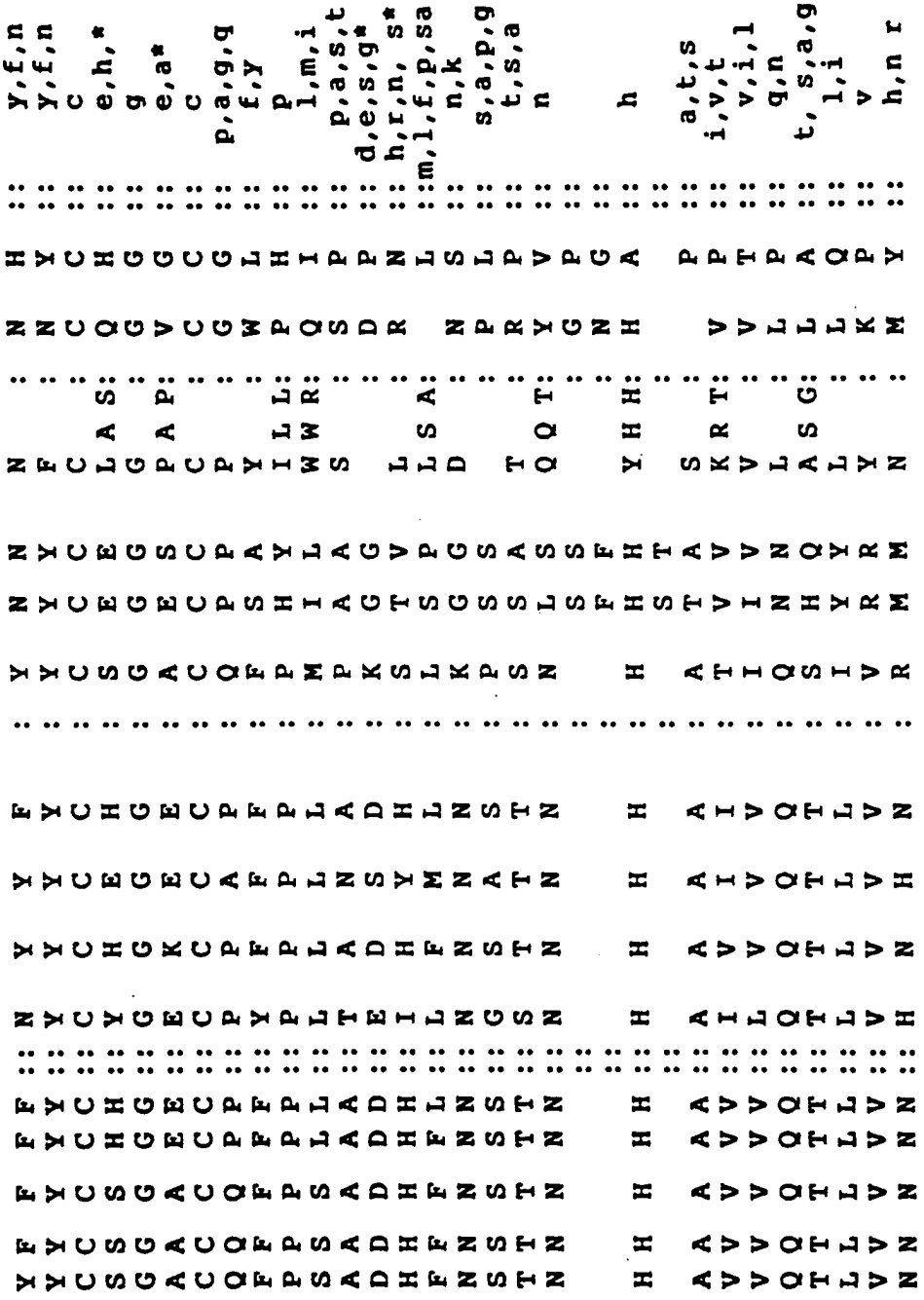


FIG. 18-2

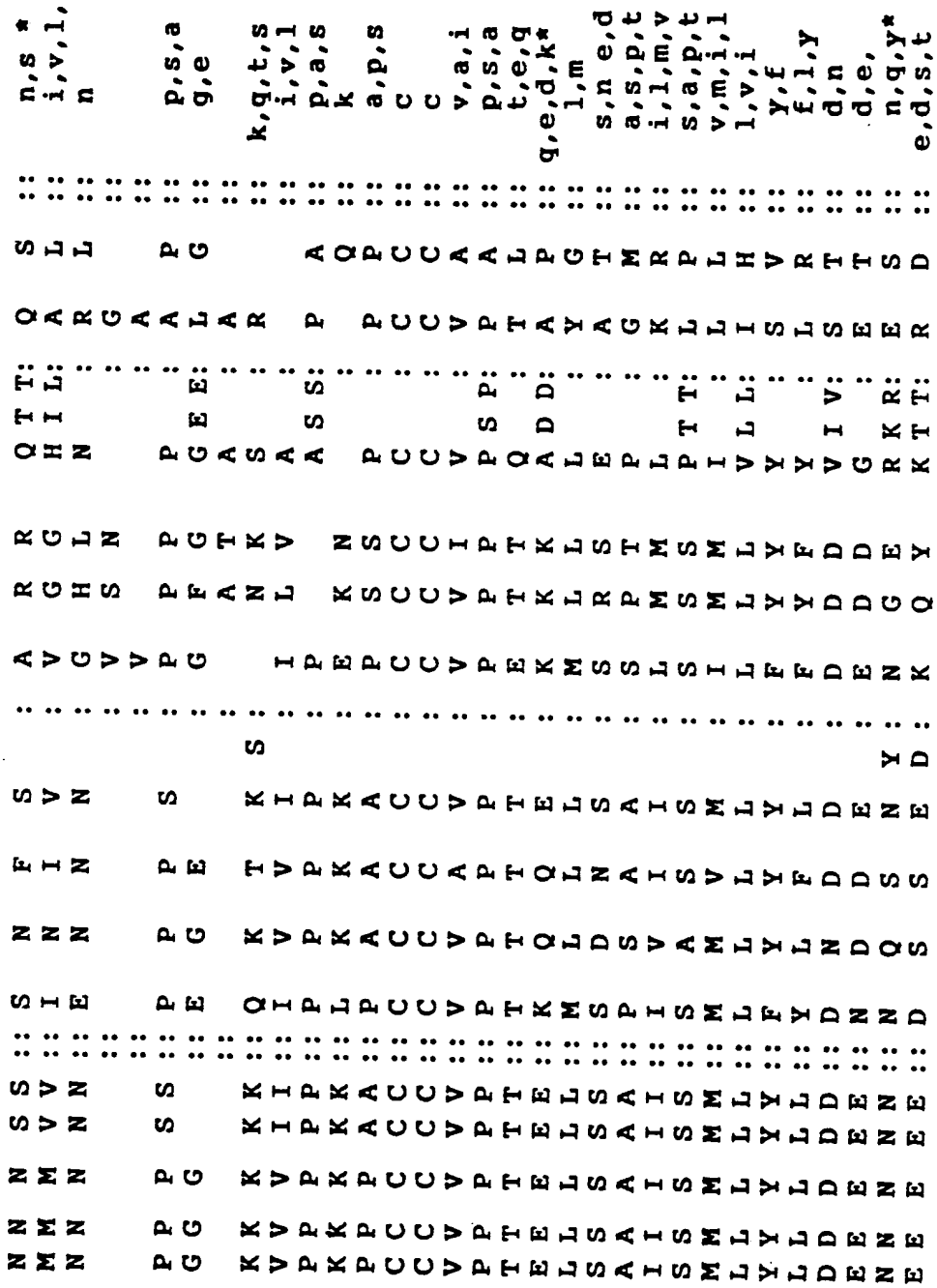


FIG. 18-3

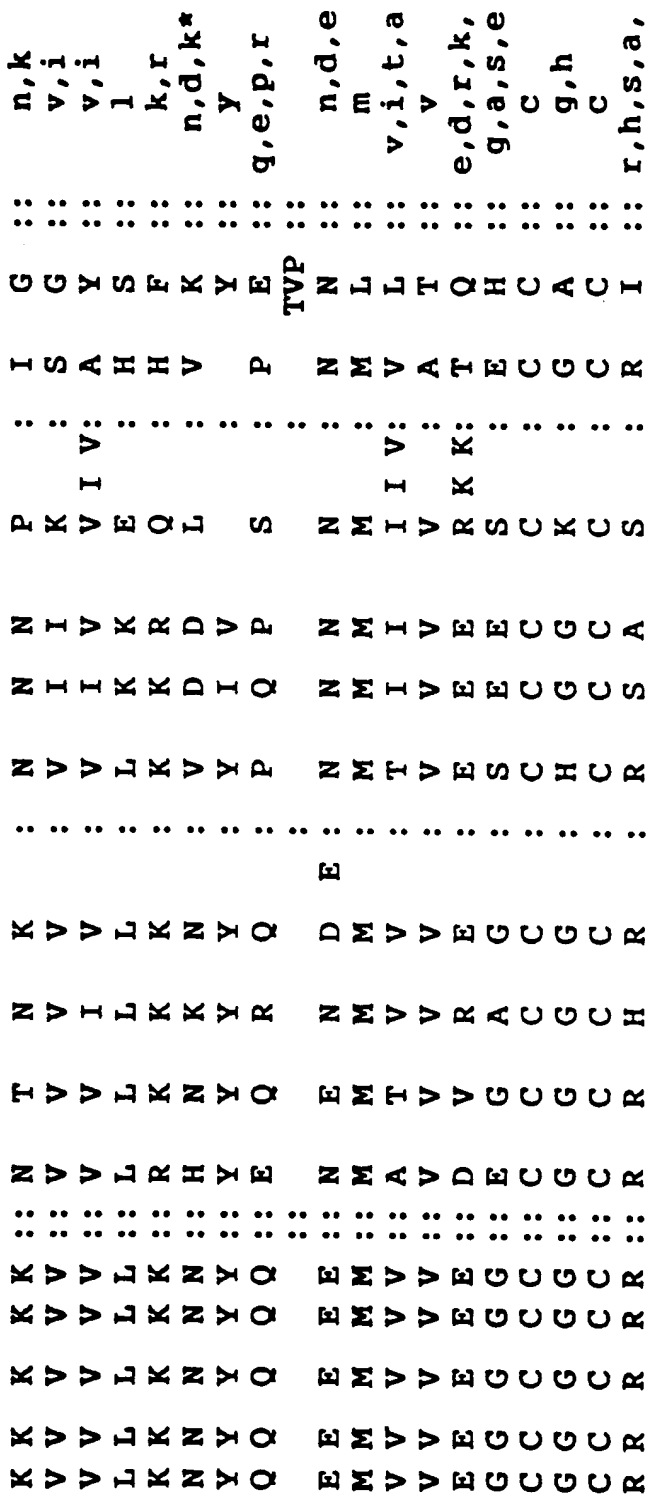


FIG. 18-4

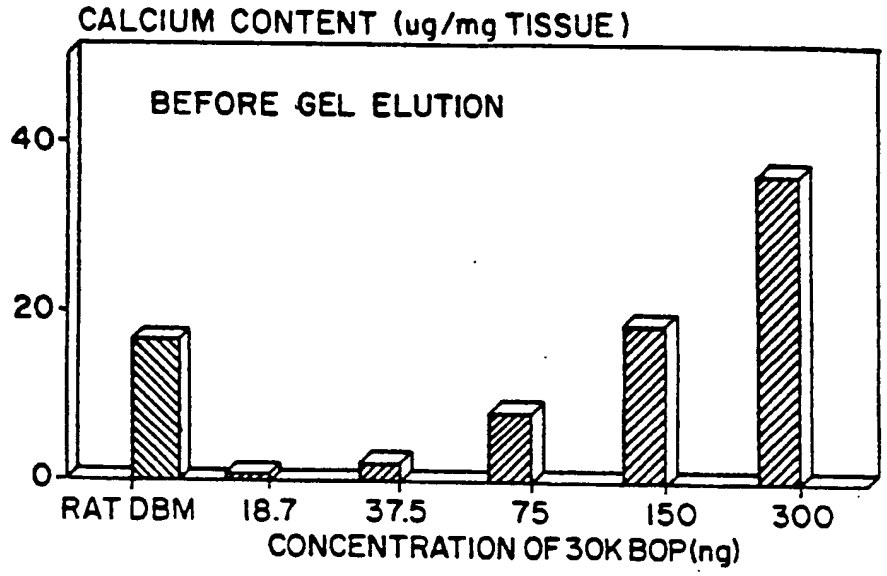


FIG. 20A

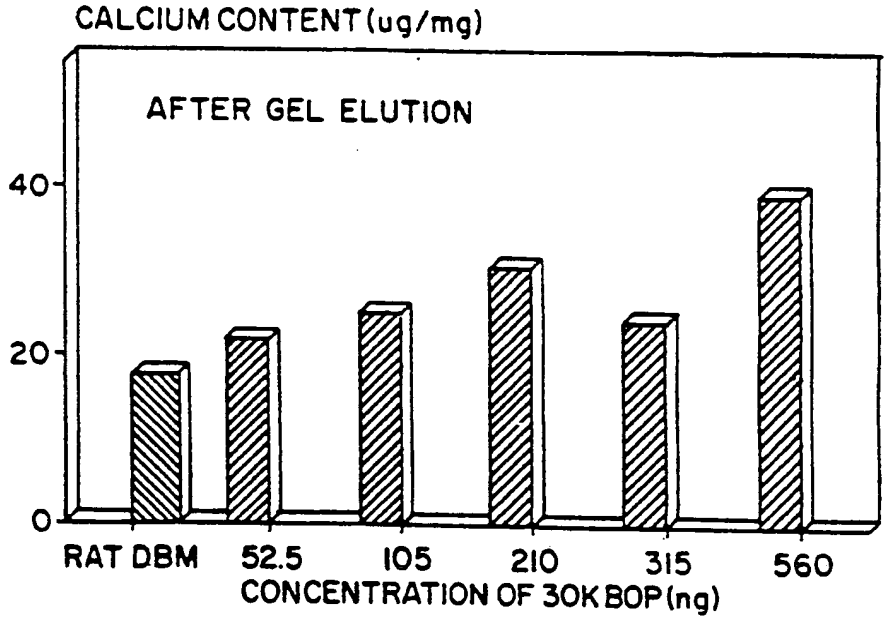


FIG. 20B

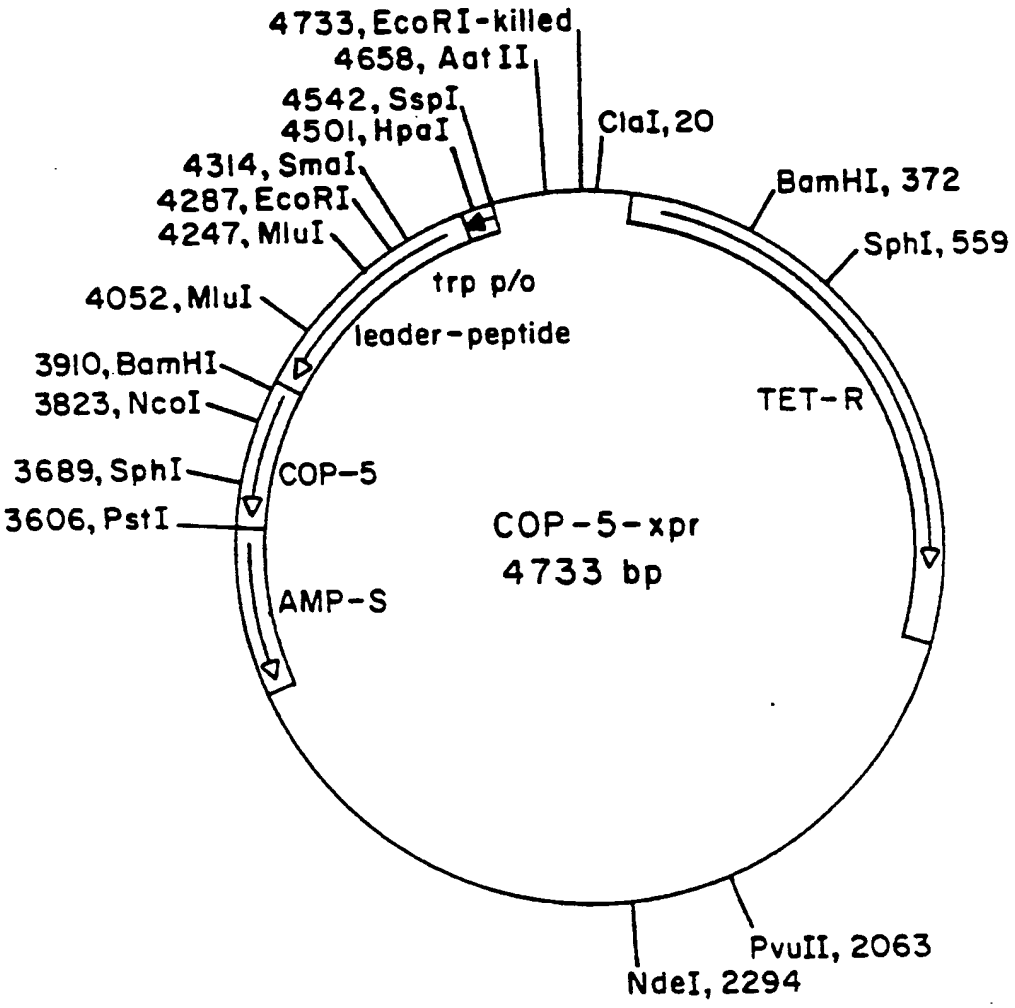

4733, EcoRI-killed
4658, AatII
4542, SspI
4501, HpaI
4314, SmaI
4287, EcoRI
4247, MluI
4052, MluI
3910, BamHI
3823, NcoI
3689, SphI
3606, PstI
ClaI, 20
BamHI, 372
SphI, 559
trp p/o
leader-peptide
TET-R
COP-5
COP-5-xpr
4733 bp
AMP-S
PvuII, 2063
NdeI, 2294

FIG. 21A

COP-5 fusion protein

```
        10         20         30         40         50
ATGAAAGCAATTTTCGTACTGAAAGGTTCACTGGACAGAGATCTGGACTC
 M  K  A  I  F  V  L  K  G  S  L  D  R  D  L  D  S
                                          BglII

        60         70         80         90        100
TCGTCTGGATCTGGACGTTCGTACCGACCACAAAGACCTGTCTGATCACC
  R  L  D  L  D  V  R  T  D  H  K  D  L  S  D  H

       110        120        130        140        150
TGGTTCTGGTCGACCTGGCTCGTAACGACCTGGCTCGTATCGTTACTCCC
L  V  L  V  D  L  A  R  N  D  L  A  R  I  V  T  P
       SalI                                      Sma

       160        170        180        190        200
GGGTCTCGTTACGTTGCGGATCTGGAATTCATGGCTGACAACAAATTCAA
 G  S  R  Y  V  A  D  L  E  F  M  A  D  N  K  F  N
I                      EcoRI

       210        220        230        240        250
CAAGGAACAGCAGAACGCGTTCTACGAGATCTTGCACCTGCCGAACCTGA
  K  E  Q  Q  N  A  F  Y  E  I  L  H  L  P  N  L
             MluI        BglII    BspMI+

       260        270        280        290        300
ACGAAGAGCAGCGTAACGGCTTCATCCAAAGCTTGAAGGATGAGCCCTCT
N  E  E  Q  R  N  G  F  I  Q  S  L  K  D  E  P  S
                         HindIII

       310        320        330        340        350
CAGTCTGCGAATCTGCTAGCGGATGCCAAGAAACTGAACGATGCGCAGGC
 Q  S  A  N  L  L  A  D  A  K  K  L  N  D  A  Q  A
             NheI                          FspI

       360        370        380        390        400
ACCGAAATCGGATCAGGGGCAATTCATGGCTGACAACAAATTCAACAAGG
  P  K  S  D  Q  G  Q  F  M  A  D  N  K  F  N  K

       410        420        430        440        450
AACAGCAGAACGCGTTCTACGAGATCTTGCACCTGCCGAACCTGAACGAA
E  Q  Q  N  A  F  Y  E  I  L  H  L  P  N  L  N  E
       MluI        BglII    BspMI+

       460        470        480        490        500
GAGCAGCGTAACGGCTTCATCCAAAGCTTGAAGGATGAGCCCTCTCAGTC
 E  Q  R  N  G  F  I  Q  S  L  K  D  E  P  S  Q  S
                   HindIII
```

FIG. 21B-1

```
       510        520        530        540        550
TGCGAATCTGCTAGCGGATGCCAAGAAACTGAACGATGCGCAGGCACCGA
  A  N  L  L  A  D  A  K  K  L  N  D  A  Q  A  P
        NheI                          FspI

       560        570        580        590        600
AGGATCCTAATGGGCTGTACGTCGACTTCAGCGACGTGGGCTGGGACGAC
K  D  P  N  G  L  Y  V  D  F  S  D  V  G  W  D  D
 BamHI              SalI

       610        620        630        640        650
TGGATTGTGGCCCCACCAGGCTACCAGGCCTTCTACTGCCATGGCGAATG
 W  I  V  A  P  P  G  Y  Q  A  F  Y  C  H  G  E  C
                          StuI          NcoI   BsmI+

       660        670        680        690        700
CCCTTTCCCGCTAGCGGATCACTTCAACAGCACCAACCACGCCGTGGTGC
  P  F  P  L  A  D  H  F  N  S  T  N  H  A  V  V
        NheI                               DraIII
                                           PflMI

       710        720        730        740        750
AGACCCTGGTGAACTCTGTCAACTCCAAGATCCCTAAGGCTTGCTGCGTG
Q  T  L  V  N  S  V  N  S  K  I  P  K  A  C  C  V
                                   MstII

       760        770        780        790        800
CCCACCGAGCTGTCCGCCATCAGCATGCTGTACCTGGACGAGAATGAGAA
 P  T  E  L  S  A  I  S  M  L  Y  L  D  E  N  E  K
                      SphI

       810        820        830        840        850
GGTGGTGCTGAAGAACTACCAGGAGATGGTAGTAGAGGGCTGCGGCTGCC
  V  V  L  K  N  Y  Q  E  M  V  V  E  G  C  G  C
                   PflMI

       860
GCTAACTGCAG
R  *
     PstI
```

FIG. 21B-2

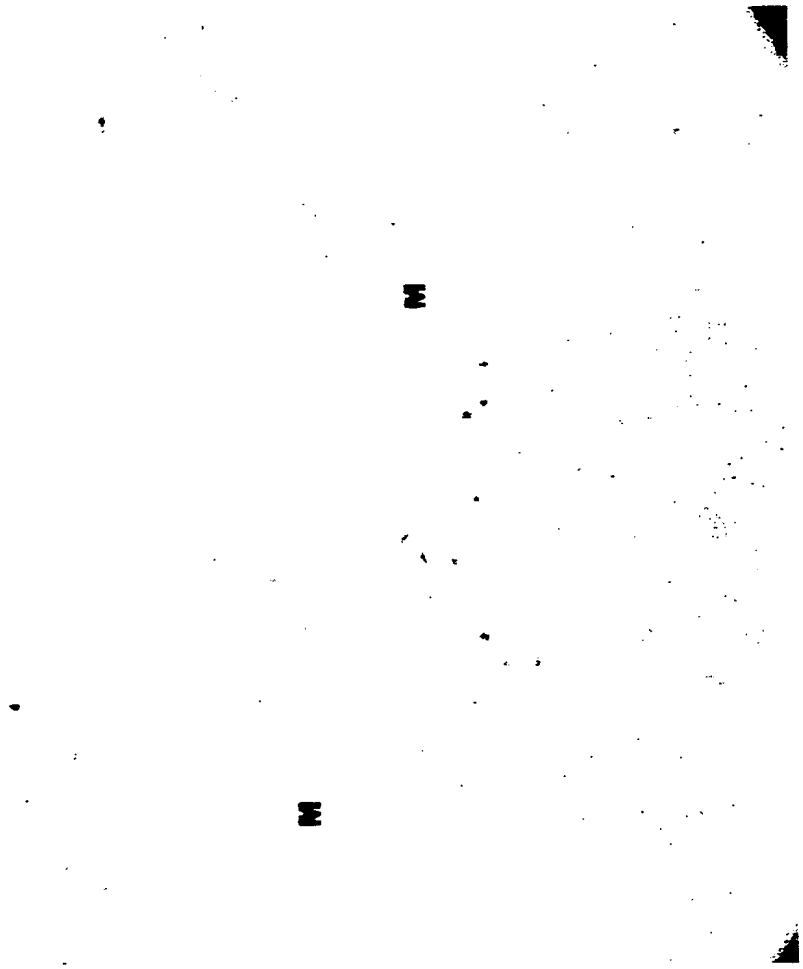


FIG. 22A

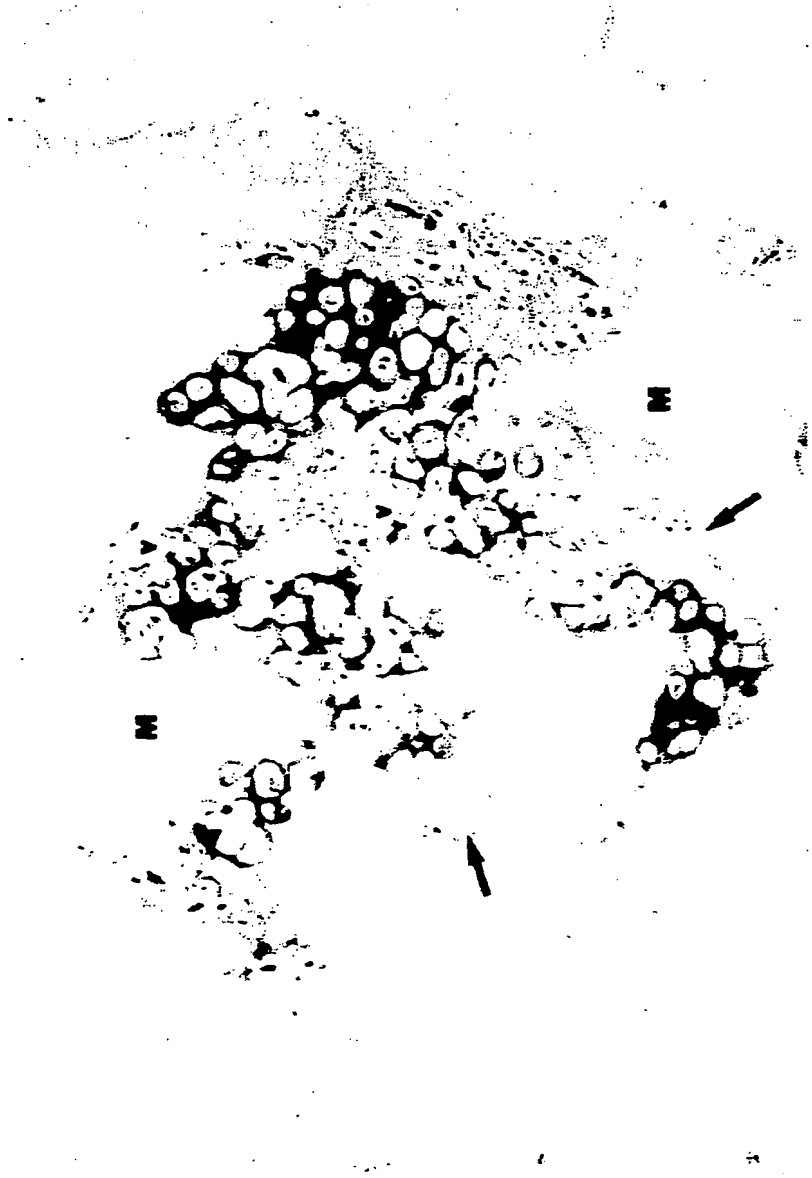


FIG. 22B

OSTEOGENIC PROTEINS

CROSS REFERENCE TO RELATED APPLICATION

This is a continuation of copending application(s) Ser. No. 07/621,988 filed on Dec. 4, 1990, now abandoned, which is a division, of application Ser. No. 315,342, filed Feb. 23, 1989 and now U.S. Pat. No. 5,011,691, which is a continuation-in-part of copending application Ser. No. 232,630, entitled "Osteogenic Devices", filed Aug. 15, 1988, now abandoned, which is a continuation-in-part of copending application Ser. No. 179,406, entitled "Osteogenic Devices", filed Apr. 8, 1988 and now U.S. Pat. No. 4,968,590.

BACKGROUND OF THE INVENTION

This invention relates to osteogenic devices, to genes encoding proteins which can induce osteogenesis in mammals and methods for their production using recombinant DNA techniques, to synthetic forms of osteogenic protein, to a method of reproducibly purifying osteogenic protein from mammalian bone, to matrix materials which act as a carrier to induce osteogenesis in mammals, and to bone and cartilage repair procedures using the osteogenic device.

Mammalian bone tissue is known to contain one or more proteinaceous materials, presumably active during growth and natural bone healing, which can induce a developmental cascade of cellular events resulting in endochondral bone formation. This active factor (or factors) has variously been referred to in the literature as bone morphogenetic or morphogenic protein, bone inductive protein, osteogenic protein, osteogenin, or osteoinductive protein.

The developmental cascade of bone differentiation consists of chemotaxis of mesenchymal cells, proliferation of progenitor cells, differentiation of cartilage, vascular invasion, bone formation, remodeling, and finally marrow differentiation (Reddi (1981) Collagen Rel. Res. 1:209-226).

Though the precise mechanisms underlying these phenotypic transformations are unclear, it has been shown that the natural endochondral bone differentiation activity of bone matrix can be dissociatively extracted and reconstituted with inactive residual collagenous matrix to restore full bone induction activity (Sampath and Reddi, (1981) Proc. Natl Acad. Sci. USA 78:7599-7603). This provides an experimental method for assaying protein extracts for their ability to induce endochondral bone in vivo.

This putative bone inductive protein has been shown to have a molecular mass of less than 50 kilodaltons (kD). Several species of mammals produce closely related protein as demonstrated by cross species implant experiments (Sampath and Reddi (1983) Proc. Natl. Acad Sci. USA 80:6591-6595).

The potential utility of these proteins has been widely recognized. It is contemplated that the availability of the pure protein would revolutionize orthopedic medicine, certain types of plastic surgery, and various periodontal and craniofacial reconstructive procedures.

The observed properties of these protein fractions have induced an intense research effort in various laboratories directed to isolating and identifying the pure factor or factors responsible for osteogenic activity. The current state of the art of purification of osteogenic protein from mammalian bone is disclosed by Sampath et al. (*Proc. Natl.* Acad. Sci. USA (1987) 80). Urist et al. (Proc. Soc. Exp. Biol. Med. (1984) 173:194-199) disclose a human osteogenic protein fraction which was extracted from demineralized cortical bone by means of a calcium chloride-urea inorganic-organic solvent mixture, and retrieved by differential precipitation in guanidine-hydrochloride and preparative gel electrophoresis. The authors report that the protein fraction has an amino acid composition of an acidic polypeptide and a molecular weight in a range of 17-18 kD.

Urist et al. (Proc. Natl. Acad. Sci. USA (1984) 81:371-375) disclose a bovine bone morphogenetic protein extract having the properties of an acidic polypeptide and a molecular weight of approximately 18 kD. The authors reported that the Protein was present in a fraction separated by hydroxyapatite chromatography, and that it induced bone formation in mouse hindquarter muscle and bone regeneration in trephine defects in rat and dog skulls. Their method of obtaining the extract from bone results in ill-defined and impure preparations.

European Patent Application Serial No. 148,155, published Oct. 7, 1985, purports to disclose osteogenic proteins derived from bovine, porcine, and human origin. One of the proteins, designated by the inventors as a P3 protein having a molecular weight of 22-24 kD, is said to have been purified to an essentially homogeneous state. This material is reported to induce bone formation when implanted into animals.

International Application No. PCT/087/01537, published Jan. 14, 1988, discloses an impure fraction from bovine bone which has bone induction qualities. The named applicants also disclose putative bone inductive factors produced by recombinant DNA techniques. Four DNA sequences were retrieved from human or bovine genomic or cDNA libraries and apparently expressed in recombinant host cells. While the applicants stated that the expressed proteins may be bone morphogenic proteins, bone induction was not demonstrated, suggesting that the recombinant proteins are not osteogenic. See also Urist et al:, EP 0,212,474 entitled Bone Morphogenic Agents.

Wang et al. (Proc. Nat. Acad. Sci. USA (1988) 85: 9484-9488) discloses the purification of a bovine bone morphogenetic protein from guanidine extracts of demineralized bone having cartilage and bone formation activity as a basic protein corresponding to a molecular weight of 30 kD determined from gel elution. Purification of the protein yielded proteins of 30, 18 and 16 kD which, upon separation, were inactive. In view of this result, the authors acknowledged that the exact identity of the active material had not been determined.

Wozney et al. (Science (1988) 242: 1528-1534) discloses the isolation of full-length cDNA's encoding the human equivalents of three polypeptides originally purified from bovine bone. The authors report that each of the three recombinantly expressed human proteins are independently or in combination capable of inducing cartilage formation. No evidence of bone formation is reported.

It is an object of this invention to provide osteogenic devices comprising matrices containing dispersed osteogenic protein capable of bone induction in allogenic and xenogenic implants. Another object is to provide a reproducible method of isolating osteogenic protein from mammalian bone tissue. Another object is to characterize the protein responsible for osteogenesis. An-

other object is to provide natural and recombinant osteogenic proteins capable of inducing endochondral bone formation in mammals, including humans. Yet another object is to provide genes encoding native and non-native osteogenic proteins and methods for their production using recombinant DNA techniques. Another object is to provide novel biosynthetic forms of osteogenic proteins and a structural design for novel, functional osteogenic proteins. Another object is to provide a suitable deglycosylated collagenous bone matrix as a carrier for osteogenic protein for use in xenogenic implants. Another object is to provide methods for inducing cartilage formation.

These and other objects and features of the invention will be apparent from the description, drawings, and claims which follow.

SUMMARY OF THE INVENTION

This invention involves osteogenic devices which, when implanted in a mammalian body, can induce at the locus of the implant the full developmental cascade of endochondral bone formation and bone marrow differentiation. Suitably modified as disclosed herein, the devices also may be used to induce cartilage formation. The devices comprise a carrier material, referred to herein as a matrix, having the characteristics disclosed below, containing dispersed osteogenic protein either in its native form or in the form of a biosynthetic construct.

A key to these developments was the elucidation of amino acid sequence and structure data of native osteogenic protein. A protocol was developed which results in retrieval of active, substantially pure osteogenic protein from mammalian bone. Investigation of the properties and structure of the native form osteogenic protein then permitted the inventors to develop a rational design for non-native forms, i.e., forms never before known in nature, capable of inducing bone formation. As far as applicants are aware, the constructs disclosed herein constitute the first instance of the design of a functional, active protein without preexisting knowledge of the active region of a native form nucleotide or amino acid sequence.

A series of consensus DNA sequences were designed with the goal of producing an active osteogenic protein. The sequences were based on partial amino acid sequence data obtained from the natural source product and on observed homologies with unrelated genes reported in the literature, or the sequences they encode, having a presumed or demonstrated developmental function. Several of the biosynthetic consensus sequences have been expressed as fusion proteins in procaryotes, purified, cleaved, refolded, combined with a matrix, implanted in an established animal model, and shown to have endochondral bone-inducing activity. The currently preferred active totally biosynthetic proteins comprise two synthetic sequences designated COP5 and COP7. The amino acid sequences of these proteins are set forth below.

```
                1         10        20        30        40
COP5            LYVDFS—DVGWDDWIVAPPGYQAFYCHGECPFPLAD

                          50        60        70
HFNSTN---H—AVVQTLVNSVNSKI---PKACCVPTELSA

                80        90        100
ISMLYLDENEKVVLKNYQEMVVEGCGCR

                1         10        20        30        40
COP7            LYVDFS—DVGWNDWIVAPPGYHAFYCHGECPFPLAD

                          50        60        70
HLNSTN---H—AVVQTLVNSVNSKI---PKACCVPTELSA

                80        90        100
ISMLYLDENEKVVLKNYQEMVVEGCGCR
```

In these sequences and all other amino acid sequences disclosed herein, the dashes (—) are used as fillers only to line up comparable sequences in related proteins, and have no other function. Thus, amino acids 46–50 of COP7, for example, are NHAVV. Also, the numbering of amino acids is selected solely for purposes of facilitating comparisons between sequences. Thus, for example, the DF residues numbered at 9 and 10 of COP5 and COP7 may comprise residues, e.g., 35 and 36, of an osteogenic protein embodying invention.

Thus, in one aspect, the invention comprises a protein comprising an amino acid sequence sufficiently duplicative of the sequence of COP5 or COP7 such that it is capable of inducing endochondral bone formation when properly folded and implanted in a mammal in association with a matrix. Some of these sequences induce cartilage, but not bone. Also, the bone forming materials may be used to produce cartilage if implanted in an avascular locus, or if an inhibitor to full bone development is implanted together with the active protein. Thus, in another aspect, the invention comprises a protein less than about 200 amino acids long in a sequence sufficiently duplicative of the sequence of COP5 or COP7 such that it is capable at least of cartilage formation when properly folded and implanted in a mammal in association with a matrix.

In one preferred aspect, these proteins comprise species of the generic amino acid sequences:

```
1         10        20        30        40        50
LXVXFXDXGWXXWXXXPXGXXAXYCXGXCXXPXXXXXXXXNHAXX

          60        70        80        90        100
QXXVXXXNXXXXPXXCCXPXXXXXXXXLXXXXXXXVXLXXYXXMXVXXCXCX
or
```

-continued

```
1          10          20          30          40          50
CXXXXLXVXFXDXGWXXWXXXPXGXXAXYCXGXCXXPXXXXXXXXNHAXX

          60          70          80          90          100
QXXVXXXNXXXXPXXCCXPXXXXXXXXLXXXXXXXVXLXXYXXMXVXXCXCX
```

where the letters indicate the amino acid residues of standard single letter code, and the Xs represent amino acid residues. Preferred amino acid sequences within the foregoing generic sequences are:

```
1          10          20          30          40          50
      LYVDFRDVGWNDWI VAPPGYHAFYCHGECPFPL ADHL NS TNHAI V
       K S S L   QE  VI S E FD Y   E A AY MPESMKAS     VI
       F E K I   DN        L     N  S    Q  I TK F  P     TL
            A    S         K

          60          70          80          90          100
QTLVNS VNPGKI PKACCVPTELS AI SMLYLDENENVVLKNYQDMVVEGCGCR
 SI  HAI  SEQV EP   A  EQMNS LAI  FFNDQDK  I  RK EE  T  DA  H  H
     RF     T    S      K  DPV V   Y  N  S        H  RN     RS
      N     S                                K           P        E
```

and

```
1          10          20          30          40          50
CKRHPLYVDFRDVGWNDWI VAPPGYHAFYCHGECPFPL ADHL NS TNHAI V
 RRRS  K  S  S  L   QE  VI S  E  FD  Y   E  A  AY  MPESMKAS     VI
   KE  F  E  K  I   DN        L      N   S     Q   I TK  F  P     TL
    Q       A      S         K

          60          70          80          90          100
QTLVNS VNPGKI PKACCVPTELS AI SMLYLDENENVVLKNYQDMVVEGCGCR
 SI  HAI  SEQV EP   A  EQMNS LAI  FFNDQDK  I  RK EE  T  DA  H  H
     RF     T    S      K  DPV V   Y  N  S        H  RN     RS
      N     S                                K           P        E
```

wherein each of the amino acids arranged vertically at each position in the sequence may be used alternatively in various combinations. Note that these generic sequences have 6 and preferably 7 cysteine residues where inter- or intramolecular disulfide bonds can form, and contain other critical amino acids which influence the tertiary structure of the proteins. These generic structural features are found in previously published sequences, none of which have been described as capable of osteogenic activity, and most of which never have been linked with such activity.

Particular useful sequences include:

```
                1          10          20          30          40
Vgl             CKKRHLYVEFK—DVGWQNWVIAPQGYMANYCYGECPYPLTE

                          50          60          70
                ILNGSN---H—AILQTLVHSIEPED—IPLPCCVPTKMSP

                     80          90          100
                ISMLFYDNNDNVVLRHYENMAVDECGCR

                1          10          20          30          40
DPP             CRRHSLYVDFS—DVGWDDWIVAPLGYDAYYCHGKCPFPLAD

                          50          60          70
                HFNSTN---H—AVVQTLVNNNNPGK—VPKACCVPTQLDS

                     80          90          100
                VAMLYLNDQSTVVLKNYQEMTVVGCGCR

                                                      -5
                                                       HQRQA

                1          10          20          30          40
OP1             CKKHELYVSFR—DLGWQDWIIAPEGYAAYYCEGECAFPLNS

                          50          60          70
                YMNATN---H—AIVQTLVHFINPET—VPKPCCAPTQLNA

                     80          90          100
                ISVLYFDDSSNVILKKYRNMVVRACGCH

                1          10          20          30          40
CBMP-2a         CKRHPLYVDFS—DVGWNDWIVAPPGYHAFYCHGECPFPLAD

                          50          60          70
                HLNSTN---H—AIVQTLVNSVNS—K—IPKACCVPTELSA

                     80          90          100
                ISMLYLDENEKVVLKNYQDMVVEGCGCR
```

-continued

```
          1         10                  20          30          40
CBMP-2b   CRRHSLYVDFS—DVGWNDWIVAPPGYQAFYCHGDCPFPLAD

                         50          60          70
          HLNSTN---H—AIVQTLVNSVNS—S—IPKACCVPTELSA

               80          90          100
          ISMLYLDEYDKVVLKNYQEMVVEGCGCR

          1         10                  20          30          40
CBMP-3    CARRYLKVDFA—DIGWSEWIISPKSFDAYYCSGACQFPMPK

                         50          60          70
          SLKPSN---H—ATIQSIVRAVGVVPGIPEPCCVPEKMSS

               80          90          100
          LSILFFDENKNVVLKVYPNMTVESCACR

          1         10                  20          30          40
COP1            LYVDFQRDVGWDDWIIAPVDFDAYYCSGACQFPSAD

                         50          60          70
          HFNSTN---H—AVVQTLVNNMNPGK—VPKPCCVPTELSA

               80          90          100
          ISMLYLDENSTVVLKNYQEMTVVGCGCR

          1         10                  20          30          40
COP3            LYVDFQRDVGWDDWIVAPPGYQAFYCSGACQFPSAD

                         50          60          70
          HFNSTN---H—AVVQTLVNNMNPGK—VPKPCCVPTELSA

               80          90          100
          ISMLYLDENEKVVLKNYQEMVVEGCGCR

          1         10                  20          30          40
COP4            LYVDFS—DVGWDDWIVAPPGYQAFYCSGACQFPSAD

                         50          60          70
          HFNSTN---H—AVVQTLVNNMNPGK—VPKPCCVPTELSA

               80          90          100
          ISMLYLDENEKVVLKNYQEMVVEGCGCR

                                        -10
                                      PKHHSQRARKKNKN

          1         10                  20          30          40
COP16     CRRHSLYVDFS—DVGWNDWIVAPPGYQAFYCHGECPFPLAD

                         50          60          70
          HFNSTN---H—AVVQTLVNSVNSKI---PKACCVPTELSA

               80          90          100
          ISMLYLDENEKVVLKNYQEMVVEGCGCR
```

Vgl is a known Xenopus sequence heretofore not associated with bone formation. DPP is an amino acid sequence encoded by a drosophila gene responsible for development of the dorsoventral pattern. OP1 is a region of a natural sequence encoded by exons of a genomic DNA sequence retrieved by applicants. The CBMPs are amino acid sequences comprising subparts of mammalian proteins encoded by genomic DNAs and cDNAs retrieved by applicants. The COPs are biosynthetic protein sequences expressed by novel consensus gene constructs, designed using the criteria set forth herein, and not yet found in nature.

These proteins are believed to dimerize during refolding. They appear not to be active when reduced. Various combinations of species of the proteins, i.e., heterodimers, have activity, as do homodimers. As far as applicants are aware, the COP5 and COP7 constructs constitute the first instances of the design of a bioactive protein without preexisting knowledge of the active region of a native form nucleotide or amino acid sequence.

The invention also provides native forms of osteogenic protein, extracted from bone or produced using recombinant DNA techniques. The substantially pure osteogenic protein may include forms having varying glycosylation patterns, varying N-termini, a family of related proteins having regions of amino acid sequence homology, and active truncated or mutated forms of native protein, no matter how derived. The osteogenic protein in its native form is glycosylated and has an apparent molecular weight of about 30 kD as determined by SDS-PAGE. When reduced, the 30 kD protein gives rise to two glycosylated polypeptide chains having apparent molecular weights of about 16 kD and 18 kD. In the reduced state, the 30 kD protein has no detectable osteogenic activity. The deglycosylated protein, which has osteogenic activity, has an apparent molecular weight of about 27 kD. When reduced, the 27 kD protein gives rise to the two deglycosylated polypeptides have molecular weights of about 14 kD to 16 kD.

Analysis of digestion fragments indicate that the native 30 kD osteogenic protein contains the following amino acid sequences (question marks indicate undetermined residues):

(1) S-F-D-A-Y-Y-C-S-G-A-C-Q-F-P-M-P-K;

(2) S-L-K-P-S-N-Y-A-T-I-Q-S-I-V;

(3) A-C-C-V-P-T-E-L-S-A-I-S-M-L-Y-L-D-E-N-E-K;
(4) M-S-S-L-S-I-L-F-F-D-E-N-K;
(5) S-Q-E-L-Y-V-D-F-Q-R;
(6) F-L-H-C-Q-F-S-E-R-N-S;
(7) T-V-G-Q-L-N-E-Q-S-S-E-P-N-I-Y;
(8) L-Y-D-P-M-V-V;
(9) V-G-V-V-P-G-I-P-E-P-C-C-V-P-E;
(10) V-D-F-A-D-I-G;
(11) V-P-K-P-C-C-A-P-T;
(12) I-N-I-A-N-Y-L;
(13) D-N-H-V-L-T-M-F-P-I-A-I-N;
(14) D-E-Q-T-L-K-K-A-R-R-K-Q-W-I-?-P;
(15) D-I-G-?-S-E-W-I-I-?-P;
(16) S-I-V-R-A-V-G-V-V-P-G-I-P-E-P-?-?-V;

ing, and expression of native genes which encode osteogenic proteins. When properly modified after translation, incorporated in a suitable matrix, and implanted as disclosed herein, these proteins are operative to induce formation of cartilage and endochondral bone.

The consensus DNA sequences are also useful as probes for extracting genes encoding osteogenic protein from genomic and cDNA libraries. One of the consensus sequences has been used to isolate a heretofore unidentified genomic DNA sequence, portions of which when ligated encode a protein having a region capable of inducing endochondral bone formation. This protein, designated OP1, has an active region having the sequence set forth below.

```
                1          10         20         30         40
OP1             LYVSFR--DLGWQDWIIAPEGYAAYYCEGECAFPLNS

                          50         60         70
YMNATN---H--AIVQTLVHFINPET--VPKPCCAPTQLNA

          80         90         100
ISVLYFDDSSNVILKKYRNMVVRACGCH
```

A longer active sequence is:

```
                                                    -5
                                                     HQRQA
                1          10         20         30         40
OP1             CKKHELYVSFR--DLGWQDWIIAPEGYAAYYCEGECAFPLNS

                          50         60         70
YMNATN---H--AIVQTLVHFINPET--VPKPCCAPTQLNA

          80         90         100
ISVLYFDDSSNVILKKYRNMVVRACGCH
```

(17) D-?-I-V-A-P-P-Q-Y-H-A-F-Y;
(18) D-E-N-K-N-V-V-L-K-V-Y-P-N-M-T-V-E;
(19) S-Q-T-L-Q-F-D-E-Q-T-L-K-?-A-R-?-K-Q;
(20) D-E-Q-T-L-K-K-A-R-R-K-Q-W-I-E-P-R-N-?-A-R-R-Y-L;
(21) A-R-R-K-Q-W-I-E-P-R-N-?-A-?-R-Y-?-?-V-D; and
(22) R-?-Q-W-I-E-P-?-N-?-A-?-?-Y-L-K-V-D-?-A-?-?-G.

The substantially pure (i.e., free of contaminating proteins having no osteoinductive activity) osteogenic proteins and the synthetics are useful in clinical applications in conjunction with a suitable delivery or support system (matrix). The matrix is made up of particles or porous materials. The pores must be of a dimension to permit progenitor cell migration and subsequent differentiation and proliferation. The particle size should be within the range of 70–850 mm, preferably 70–420 mm. It may be fabricated by close packing particulate material into a shape spanning the bone defect, or by otherwise structuring as desired a material that is biocompatible (non-inflammatory) and, biodegradable in vivo to serve as a "temporary scaffold" and substratum for recruitment of migratory progenitor cells, and as a base for their subsequent anchoring and proliferation. Currently preferred carriers include particulate, demineralized, guanidine extracted, species-specific (allogenic) bone, and particulate, deglycosglated, protein extracted, demineralized, xenogenic bone. Optionally, such xenogenic bone powder matrices also may be treated with proteases such as trypsin. Other useful matrix materials comprise collagen, homopolymers and copolymers of glycolic acid and lactic acid, hydroxyapatite, tricalcium phosphate and other calcium phosphates.

The availability of the protein in substantially pure form, and knowledge of its amino acid sequence and other structural features, enable the identification, clon-

FIG. 1A discloses the genomic DNA sequence of OP1.

The probes have also retrieved the DNA sequences identified in PCT/087/01537, referenced above, designated therein as BMPII(b) and BMPIII. The inventors herein have discovered that certain subparts of these genomic DNAs, and BMPIIa, from the same publication, when properly assembled, encode proteins (CBMPIIa, CBMPIIb, and CBMPIII) which have true osteogenic activity, i.e., induce the full cascade of events when properly implanted in a mammal leading to endochondral bone formation.

Thus, in view of this disclosure, skilled genetic engineers can design and synthesize genes or isolate genes from cDNA or genomic libraries which encode appropriate amino acid sequences, and then can express them in various types of host cells, including both procaryotes and eucaryotes, to produce large quantities of active proteins in native forms, truncated analogs, muteins, fusion proteins, and other constructs capable of inducing bone formation in mammals including humans.

The osteogenic proteins and implantable osteogenic devices enabled and disclosed herein will permit the physician to obtain optimal predictable bone formation to correct, for example, acquired and congenital craniofacial and other skeletal or dental anomalies (Glowacki et al. (1981) Lancet 1:959–963). The devices may be used to induce local endochondral bone formation in non-union fractures as demonstrated in animal tests, and in other clinical applications including periodontal applications where bone formation is required. The other potential clinical application is in cartilage repair, for example, in the treatment of osteoarthritis.

BRIEF DESCRIPTION OF THE DRAWING

The foregoing and other objects of this invention, the various features thereof, as well as the invention itself,

may be more fully understood from the following description, when read together with the accompanying drawings, in which:

FIGS. 1A-1 to FIGS. 1A-6 represents the nucleotide sequence of the genomic copy of osteogenic protein "OP1" gene. The unknown region between 1880 and 1920 actually represents about 1000 nucleotides;

FIG. 1B is a representation of the hybridization of the consensus gene/probe to the osteogenic protein "OP1" gene;

FIGS. 2A to 2D are collection of plots of protein concentration (as indicated by optical absorption) vs elution volume illustrating the results of bovine osteogenic protein (BOP) fractionation during purification on (A) heparin-Sepharose I; (B) HAP-Ultragel; (C) sieving gel (Sephacryl 300); and (D) heparin-Sepharose II;

FIGS. 3A to 3B are photographic reproductions of a Coomassie blue stained SDS polyacrylamide gel of the osteogenic protein under non-reducing (A) and reducing (B) conditions;

FIGS. 4A to 4B are photographic reproductions of a Con A blot of an SDS polyacrylamide gel showing the carbohydrate component of oxidized (A) and reduced (B) 30 kD protein;

FIGS. 5A and 5B are photographic reproductions of an autoradiogram of an SDS polyacrylamide gel of ^{125}I-labelled glycosylated (A) and deglycosylated (B) osteogenic protein under non-reducing (1) and reducing (2) conditions;

FIGS. 6A to 6E are photographic reproductions of an autoradiogram of an SDS polyacrylamide gel of peptides produced upon the digestion of the 30 kD osteogenic protein with V-8 protease (B), Endo Lys C protease (C), pepsin (D), and trypsin (E). (A) is control;

FIGS. 7A to 7C are a collection of HPLC chromatograms of tryptic peptide digestions of 30 kD BOP (A), the 16 kD subunit (B), and the 18 kD subunit (C);

FIG. 8 is an HPLC chromatogram of an elution profile on reverse phase C-18 HPLC of the samples recovered from the second heparin-Sepharose chromatography step (see FIG. 2D). Superimposed is the percent bone formation in each fraction;

FIG. 9 is a gel permeation chromatogram of an elution profile on TSK 3000/2000 gel of the C-18 purified osteogenic peak fraction. Superimposed is the percent bone formation in each fraction;

FIG. 10 are four graphs of protein concentration (as indicated by optical absorption) vs. elution volume illustrating the results of human protein fractionation on heparin-Sepharose I (A), HAP-Ultragel (B), TSK 3000/2000 (C), and heparin-Sepharose II (D). Arrows indicate buffer changes;

FIG. 11 is a graph showing five representative dose response curves, labelled A-E, for bone-inducing activity in samples taken from the different column purification steps: reverse phase HPLC on C-18 (A), Heparin-Sepharose II (B), Sephacryl S-300 HR (C), HAP-ultragel (D), and Heparing-Sepharose I (E);

FIG. 12 is a bar graph of radiomorphometric analyses of feline bone defect repair after treatment with an osteogenic device (A), carrier control (B), and demineralized bone (C);

FIG. 13 is a schematic representation of the DNA sequence and corresponding amino acid sequence of a consensus gene/probe for osteogenic protein (COPO);

FIG. 14 is a graph of osteogenic activity, measured by calcium content vs. increasing molecular weight, showing peak bone forming activity in the 30 kD region of an SDS polyacrylamide gel;

FIG. 15 is a photographic representation of a Coomassie blue stained SDS gel showing gel purified subunits of the 30 kD protein;

FIGS. 16A and 16B are a pair of HPLC chromatograms of Endo Asp N proteinase digests of the 18 kD subunit (A) and the 16 kD subunit (B);

FIGS. 17A to 17C are three photographic representations of the histological examination of bone implants in the rat model: carrier alone (A); carrier and glycosylated osteogenic protein (B); and carrier and deglycosylated osteogenic protein (C). Arrows indicate osteoblasts;

FIGS. 18-1 to 18-4 is a comparison of the amino acid sequence of various osteogenic proteins to those of the TGF-beta family. COP1, COP3, COP4, COP5 , and COP7 are a family of analogs of synthetic osteogenic proteins developed from the consensus gene that was joined to a leader protein via a hinge region having the sequence D-P-N-G that permitted chemical cleavage at the D-P site (by acid) or N-G (by hydroxylamine) resulting in the release of the analog protein; VGI is a Xenopus protein, DPP is a Drosophila protein; OP1 is a native osteogenic protein; CBMP2a and 2b, and CBMP3 are subparts of proteins disclosed in PCT application 087/01537; MIS is Mullerian inhibitory substance; and "consensus choices" represent various substitutions of amino acids that may be made at various positions in osteogenic proteins;

FIG. 19 is a graph showing the specific activity, as measured by alkaline phosphatase activity, of osteogenic devices comprising heparin-Sepharose II-purified naturally sourced OP and allogenic (rat) bone matrix, or xenogenic (bovine and deglycosylated bovine) bone matrix;

FIGS. 20A and 20B are bar graphs showing the specific activity of naturally sourced OP before gel elution (A), and after gel elution (B), as measured by calcium content vs. increasing concentrations of proteins (dose curve, in ng);.

FIG. 21A is an *E. coli* expression vector containing a gene of an osteogenic protein fused to a leader protein;

FIGS. 21B-1 to 21B-2 is the DNA sequence comprising a modified trp-LE leader, two Fb domains of protein A, an ASP-PRO cleavage site, and the COP5 sequence;

FIGS. 22A and 22B are photomicrographs of implants showing the histology (day 12) of COP5 active recombinant protein. A is a control (rat matrix alone, 25 mg). B is rat matrix plus COP5, showing +++ cartilage formation and ++ bone formation (see key infra). Similar results are achieved with COP7.

DESCRIPTION

Purification protocols have been developed which enable isolation of the osteogenic protein present in crude protein extracts from mammalian bone. While each of the separation steps constitute known separation techniques, it has been discovered that the combination of a sequence of separations exploiting the protein's affinity for heparin and for hydroxyapatite (HAP) in the presence of a denaturant such as urea is key to isolating the pure protein from the crude extract. These critical separation steps are combined with separations on hydrophobic media, gel exclusion chromatography, and elution form SDS PAGE.

The isolation procedure enables the production of significant quantities of substantially pure osteogenic protein from any mammalian species, provided sufficient amounts of fresh bone from the species is available. The empirical development of the procedure, coupled with the availability of fresh calf bone, has enabled isolation of substantially pure bovine osteogenic protein (BOP). BOP has been characterized significantly as set forth below; its ability to induce cartilage and ultimately endochondral bone growth in cat, rabbit, and rat have been studied; it has been shown to be able to induce the full developmental cascade of bone formation previously ascribed to unknown protein or proteins in heterogeneous bone extracts; and it may be used to induce formation of endochondral bone in orthopedic defects including non-union fractures. In its native form it is a glycosylated, dimeric protein. However, it is active in deglycosylated form. It has been partially sequenced. Its primary structure includes the amino acid sequences set forth herein.

Elucidation of the amino acid sequence of BOP enables the construction of pools of nucleic acid probes encoding peptide fragments. Also, a consensus nucleic acid sequence designed as disclosed herein based on the amino acid sequence data, inferred codons for the sequences, and observation of partial homology with known genes, also may be used as a probe. The probes may be used to isolate naturally occuring cDNAs which encode active mammalian osteogenic proteins (OP) as described below using standard hybridization methodology. The mRNAs are present in the cytoplasm of cells of various species which are known to synthesize osteogenic proteins. Useful cells harboring the mRNAs include, for example, osteoblasts from bone or osteosarcoma, hypertrophic chondrocytes, and stem cells. The mRNAs can be used to produce cDNA libraries. Alternatively, relevant DNAs encoding osteogenic protein may be retrieved from cloned genomic DNA libraries from various mammalian species.

The consensus sequence described above also may be refined by comparison with the sequences present in certain regulatory genes from drosophila, xenopus, and human followed by point mutation, expression, and assay for activity. This approach has been successful in producing several active totally synthetic constructs not found in nature (as far as applicants are aware) which have true osteogenic activity.

These discoveries enable the construction of DNAs encoding totally novel, non-native protein constructs which individually, and combined are capable of producing true endochondral bone. They also permit expression of the natural material, truncated forms, muteins, analogs, fusion proteins, and various other variants and constructs, from cDNAs retrieved from natural sources or synthesized using the techniques disclosed herein using automated, commercially available equipment. The DNAs may be expressed using well established recombinant DNA technologies in procaryotic or eucaryotic host cells, and may be oxidized and refolded in vitro if necessary for biological activity.

The isolation procedure for obtaining the protein from bone, the retrieval of an osteogenic protein gene, the design and production of biosynthetics, the nature of the matrix, and other material aspects concerning the nature, utility, how to make, and how to use the subject matter claimed herein will be further understood from the following, which constitutes the best method currently known for practicing the various aspects of the invention.

I. NATURALLY SOURCED OSTEOGENIC pROTEIN

A Purification

A1. Preparation of Demineralized Bone

Demineralized bovine bone matrix is prepared by previously published procedures (Sampath and Reddi (1983) Proc. Natl. Acad. Sci. USA 80:6591–6595). Bovine diaphyseal bones (age 1–10 days) are obtained from a local slaughterhouse and used fresh. The bones are stripped of muscle and fat, cleaned of periosteum, demarrowed by pressure with cold water, dipped in cold absolute ethanol, and stored at −20° C. They are then dried and fragmented by crushing and pulverized in a large mill. Care is taken to prevent heating by using liquid nitrogen. The pulverized bone is milled to a particle size between 70–420 mm and is defatted by two washes of approximately two hours duration with three volumes of chloroform and methanol (3:1). The particulate bone is then washed with one volume of absolute ethanol and dried over one volume of anhydrous ether. The defatted bone powder (the alternative method is to obtain Bovine Cortical Bone powder (75–425 mm) from American Biomaterials) is then demineralized with 10 volumes of 0.5N HCl at 4° C. for 40 min., four times. Finally, neutralizing washes are done on the demineralized bone powder with a large volume of water.

A2. Dissociative Extraction and Ethanol Precipitation

Demineralized bone matrix thus prepared is dissociatively extracted with 5 volumes of 4M guanidine-HCl, 50 mM Tris-HCl, pH 7.0, containing protease inhibitors (5 mM benzamidine, 44 mM 6-aminohexanoic acid, 4.3 mM N-ethylmaleimide, 0.44 mM phenylmethylsulfonyfluoride) for 16 hr. at 4° C. The suspension is filtered. The supernatant is collected ultrafiltration hollow fiber membrane (Amicon, YM-10). The concentrate is centrifuged (8,000×g for 10 min. at 4° C.), and the supernatant is then subjected to ethanol precipitation. To one volume of concentrate is added five volumes of cold (−70° C.) absolute ethanol (100%), which is then kept at −70° C. for 16 hrs. The precipitate is obtained upon centrifugation at 10,000×g for 10 min. at 4° C. The resulting pellet is resuspended in 4 l of 85% cold ethanol incubated for 60 min. at −70° C. and recentrifuged. The precipitate is again resuspended in 85% cold ethanol (2 l), incubated at −70° C. for 60 min. and centrifuged. The precipitate is then lyophilized.

A3. Heparin-Sepharose Chromatography I

The ethanol precipitated, lyophilized, extracted crude protein is dissolved in 25 volumes of 6M urea, 50 mM Tris-HCl, pH 7.0 (Buffer A) containing 0.15M NaCl, and clarified by centrifugation at 8,000 ×g for 10 min. The heparin-Sepharose is column-equilibrated with Buffer A. The protein is loaded onto the column and after washing with three column volume of initial buffer (Buffer A containing 0.15M NaCl), protein is eluted with Buffer A containing 0.5M NaCl. The absorption of the eluate is monitored continuously at 280 nm. The pool of protein eluted by 0.5M NaCl (approximately 1 column volumes) is collected and stored at 4° C.

As shown in FIG. 2A, most of the protein (about 95%) remains unbound. Approximately 5% of the pro-

tein is bound to the column. The unbound fraction has no bone inductive activity when bioassayed as a whole or after a partial purification through Sepharose CL-6B.

A4. Hydroxyapaptite-Ultrogel Chromatography

The volume of protein eluted by Buffer A containing 0.5M NaCl from the heparin-Sepharose is applied directly to a column of hydroxyapaptiteultrogel (HAP-ultrogel) (LKB Instruments), equilibrated with Buffer A containing 0.5 M NaCl. The HAP-ultrogel is treated with Buffer A containing 500 mM Na phosphate prior to equilibration. The unadsorbed protein is collected as an unbound fraction, and the column is washed with three column volumes of Buffer A containing 0.5M NaCl. The column is subsequently eluted with Buffer A containing 100 mM Na phosphate (FIG. 2B).

The eluted component can induce endochondral bone as measured by alkaline phosphatase activity and histology. As the biologically active protein is bound to HAP in the presence of 6M urea and 0.5M NaCl, it is likely that the protein has an affinity for bone mineral and may be displaced only by phosphate ions.

A5. Sephacryl S-300 Gel Exclusion Chromatography

Sephacryl S-300 HR (High Resolution, 5 cm× 100 cm column) is obtained from pharmacia and equilibrated with 4M guanidine-HCl, 50 mM Tris-HCl, pH 7.0. The bound protein fraction from HA-ultrogel is concentrated and exchanged from urea to 4M guanidine-HCl, 50 mM Tris-HCl, pH 7.0 via an Amicon ultrafiltration YM-10 membrane. The solution is then filtered with Schleicher and Schuell CENTREX disposable microfilters. A sample aliquot of approximately 15 ml containing approximately 400 mg of protein is loaded onto the column and then eluted with 4M guanidine-HCl, 50 mM Tris-HCl, pH 7.0, with a flow rate of 3 ml/min; 12 ml fractions are collected over 8 hours and the concentration of protein is measured at A_{280nm} (FIG. 2C). An aliquot of the individual fractions is bioassayed for bone formation. Those fractions which have shown bone formation and have a molecular weigh less than 35 kD are pooled and concentrated via an Amicon ultrafiltration system with YM-10 membrane.

A6. Heparin-Sepharose Chromatography-II

The pooled osteo-inductive fractions obtained from gel exclusion chromatography are dialysed extensively against distilled water and then against 6M urea, 50 mM Tris-HCl, pH 7.0 (Buffer A) containing 0.1M NaCl. The dialysate is then cleared through centrifugation. The sample is applied to the heparin-sepharose column (equilibrated with the same buffer). After washing with three column volumes of initial buffer, the column is developed sequentially with Buffer B containing 0.15M NaCl, and 0.5M NaCl (FIG. 2D). The protein eluted by 0.5M NaCl is collected and dialyzed extensively against distilled water. It is then dialyzed against 30% acetonitrile, 0.1% TFA at 4° C.

A7. Reverse Phase HPLC

The protein is further purified by C-18 Vydac silica-based HPLC column chromatography (particle size 5 mm; pore size 300 A). The osteoinductive fraction obtained from heparin-sepharose-II chromatograph is loaded onto the column, and washed in 0.1% TFA, 10% acetonitrile for five min. As shown in FIG. 8, the bound proteins are eluted with a linear gradient of 10–30% acetonitrile over 15 min., 30–50% acetonitrile over 60 min, and 50–70% acetonitrile over 10 min at 22° C. with a flow rate of 1.5 ml/min and 1.4 ml samples are collected in polycarbonate tubes. Protein is monitored by absorbance at A_{214} nm. Column fractions are tested for the presence of osteoinductive activity, concanavalin A-blottable proteins and then pooled. Pools are then characterized biochemically for the presence of 30 kD protein by autoradiography, concanavalin A blotting, and Coomassie blue dye staining. They are then assayed for in vivo osteogenic activity. Biological activity is not found in the absence of 30 kD protein.

A8. Gel Elution

The glycosylated or deglycosylated protein is eluted from SDS gels (0.5 mm and 1.5 mm thickness) for further characterization. ^{125}I-labelled 30 kD protein is routinely added to each preparation to monitor yields. TABLE 1 shows the various elution buffers that have been tested and the yields of ^{125}I-labelled protein.

TABLE 1

Elution of 30 kD Protein from SDS Gel

Buffer	% Eluted 0.5 mm	% Eluted 1.5 mm
(1) dH_2O	22	
(2) 4M Guanidine-HCl, Tris-HCl, pH 7.0	2	
(3) 4M Guanidine-HCl, Tris-HCl, pH 7.0, 0.5% Triton × 100	93	52
(4) 0.1% SDS, Tris-HCl, pH 7.0	98	

TABLE 2 lists the steps used to isolate the 30 kD or deglycosylated 27 kD gel-bound protein. The standard protocol uses diffusion elution using 4M guanidine-HCl containing 0.5% Triton×100 in Tris-HCl buffer or in Tris-HCl buffer containing 0.1% SDS to achieve greater than 95% elution of the protein from the 27 or 30 kD region of the gel for demonstration of osteogenic activity in vivo as described in later section.

In order to isolate substantially purified 30 kD or deglycosylated 27 kD protein for sequencing and characterization, the following steps are mentioned in Table 2.

TABLE 2

Preparation of Gel Eluted Protein

(C-18 Pool or deglycoslated protein plus ^{125}I-labelled 30 kD protein)

1. Dry using vacuum centrifugation;
2. Wash pellet with H_2O;
3. Dissolve pellet in gel sample buffer (no reducing agent)
4. Electrophorese on pre-electrophoresed 0.5 mm mini gel;
5. Cut out 27 or 30 kD protein;
6. Elute from gel with 0.1% SDS, 50 mM Tris-HCl, pH 7.0;
7. Filter through Centrex membrane;
8. Concentrate in Centricon tube (10 kD membrane);
9. Chromatograph of TSK-3000 gel filtration column;
10. Concentrate in Centricon tube.

Chromatography in 0.1% SDS on a TSK-3000 gel filtration column is performed to separate gel impurities, such as soluble acrylamide, from the final product. The overall yield of labelled 30 kD protein from the gel elution protocol is 50–60% of the loaded sample. Most of the loss occurs in the electrophoresis step, due to protein aggregation and/or smearing. In a separate experiment, a sample of gel eluted 30 kD protein is

reduced, electrophoresed on an SDS gel, and transferred to an Immobilon membrane. The membrane is stained with Coomassie blue dye, cut into slices, and the slices are counted. Coomassie blue dye stains the 16 kD and 18 kD reduced species of the 30 kD protein almost exclusively. However, the counts showed significant smearing throughout the gel in addition to being concentrated in the 16 kD and 18 kD species. This suggests that the ^{125}I-label can exhibit anomolous behavior on SDS gels and cannot be used as an accurate marker for cold protein under such circumstances.

The yield is 0.5 to 1.0 mg substantially pure osteogenic protein per kg of bone.

A9. Isolation of the 16 kD and 18 kD Species

TABLE 3 summarizes the procedures involved in the preparation of the subunits. Approximately 10 mg of gel eluted 30 kD protein (FIG. 3) is carboxymethylated and electrophoresed on an SDS-gel. The sample contains ^{125}I-label to trace yields and to use as an indicator for slicing the 16 kD, 18 kD and non-reduceable 30K regions from the gel. FIG. 15 shows a Coomassie stained gel of aliquots of the protein isolated from the different gel slices. The slices corresponding to the 16 kD, 18 kD and non-reduceable 30 kD species contained approximately 2–3 mg, 3–4 mg, and 1–2 mg, of protein respectively, as estimated by staining intensity. Prior to SDS electrophoresis, all of the 30 kD species can be reduced to the 16 kD and 18 kD species. The nonreducible 30 kD species observed after electrophoresis appears to be an artifact resulting from the electrophoresis procedure.

TABLE 3

Isolation of the Subunits of the 30 kD protein (C-18 pool plus ^{125}I labeled 30 kD protein)
1. Electrophorese on SDS gel.
2. Cut out 30 kD protein.
3. Elute with 0.1% SDS, 50 nm Tris, pH 7.0.
4. Concentrate and wash with H_2O in Centricon tube (10 kD membranes).
5. Reduce and carboxymethylate in 1% SDS, 0.4M Tris, pH 8.5.
6. Concentrate and wash with H_2O in Centricon tube.
7. Electrophorese on SDS gel.
8. Cut out the 16 kD and 18 kD subunits.
9. Elute with 0.1% SDS, 50 mM Tris, pH 7.0.
10. Concentrate and wash with H_2O in Centricon tubes.

B. Demonstration that the 30 kD Protein is Osteogenic Protein—Biological Characterization

B1. Gel Slicing

Gel slicing experiments confirm that the isolated 30 kD protein is the protein responsible for osteogenic activity.

Gels from the last step of the purification are sliced. Protein in each fraction is extracted in 15 mM Tris-HCl, pH 7.0 containing 0.1% SDS or in buffer containing 4M guanidine-HCl, 0.5% non-ionic detergent (Triton X 100), 50 mM Tris-HCl. The extracted proteins are desalted, concentrated, and assayed for endochondral bone formation activity. The results are set forth in FIG. 14. From this Figure it is clear that the majority of osteogenic activity is due to protein at 30 kD region of the gene. Activity in higher molecular weight regions is apparently due to protein aggregation. These protein aggregates, when reduced, yields the 16 kD and 18 kD species discussed above.

B2. Con A-Sepharose Chromatography

A sample containing the 30 kD protein is solubilized using 0.1% SDS, 50 mM Tris-HCl, and is applied to a column of Con A-Sepharose equilibrated with the same buffer. The bound material is eluted in SDS Tris-HCl buffer containing 0.5M alpha-methyl mannoside. After reverse phase chromatography of both the bound and unbound fractions, Con A-bound materials, when implanted, result in extensive bone formation. Further characterization of the bound materials show a Con A-blottable 30 kD protein. Accordingly, the 30 kD glycosylated protein is responsible for the bone forming activity.

B3. Gel Permeation Chromatography

TSK-3000/2000 gel permeation chromatography in guanidine-HCl alternately is used to achieve separation of the high specific activity fraction obtained from C-18 chromatography (FIG. 9). The results demonstrate that the peak of bone inducing activity elutes in fractions containing substantially pure 30 kD protein by Coomassie blue staining. When this fraction is iodinated and subjected to autoradiography, a strong band at 30 kD accounts for 90% of the iodinated proteins. The fraction induces bone formation in vivo at a dose of 50 to 100 ng per implant.

B4. Structural Requirements for Biological Activity

Although the role of 30 kD osteogenic protein is clearly established for bone induction, through analysis of proteolytic cleavage products we have begun to search for a minimum structure that is necessary for activity in vivo. The results of cleavage experiments demonstrate that pepsin treatment fails to destroy bone inducing capacity, whereas trypsin or CNBr completely abolishes the activity.

An experiment is performed to isolate and identify pepsin digested product responsible for biological activity. Sample used for pepsin digest were 20%–30% pure. The buffer used is 0.1% TFA in water. The enzyme to substrate ratio is 1:10. A control sample is made without enzyme. The digestion mixture is incubated at room temperature for 16 hr. The digested product is then separated in 4M guanidine-HCl using gel permeation chromatography, and the fractions are prepared for in vivo assay. The results demonstrate that active fractions from gel permeation chromotography of the pepsin digest correspond to molecular weight of 8 kD–10 kD.

In order to understand the importance of the carbohydrates moiety with respect to osteogenic activity, the 30 kD protein has been chemically deglycosylated using HF (see below). After analyzing an aliquot of the reaction product by Con A blot to confirm the absence of carbohydrate, the material is assayed for its activity in vivo. The bioassay is positive (i.e., the deglycosylated protein produces a bone formation response as determined by histological examination shown in FIG. 17C), demonstrating that exposure to HF did not destroy the biological function of the protein. In addition, the specific activity of the deglycosylated protein is approximately the same as that of the native glycosylated protein.

B5. Specific Activity of BOP

Experiments were performed 1) to determine the half maximal bone-inducing activity based on calcium content of the implant; 2) to estimate proteins at nanogram levels using a gel scanning method; and 3) to establish dose for half maximal bone inducing activity for gel eluted 30 kD BOP. The results demonstrate that gel eluted substantially pure 30 kD osteogenic protein induces bone at less than 5 ng per 25 mg implant and exhibits half maximal bone differentiation activity at 20 ng per implant. The purification data suggest that osteogenic protein has been purified from bovine bone to 367,307 fold after final gel elution step with a specific activity of 47,750 bone forming units per mg of protein.

B5(a) Half Maximal Bone Differentiation Activity

The bone inducing activity is determined biochemically by the specific activity of alkaline phosphatase and calcium content of the day 12 implant. An increase in the specific activity of alkaline phosphatase indicates the onset of bone formation. Calcium content, on the other hand, is proportional to the amount of bone formed in the implant. The bone formation is therefore calculated by determining calcium content of the implant on day 12 in rats and expressed as bone forming units, which represent the amount that exhibits half maximal bone inducing activity compared to rat demineralized bone matrix. Bone induction exhibited by intact demineralized rat bone matrix is considered to be the maximal bone-differentiation activity for comparison.

B5(b) Protein Estimation Using Gel Scanning Techniques

A standard curve is developed employing known amounts of a standard protein, bovine serum albumin. The protein at varying concentration (50–300 ng) is loaded on 15% SDS gel, electrophoresed, stained in comassie and destained. The gel containing standard proteins is scanned at predetermined settings using a gel scanner at 580 nm. The area covered by the protein band is calculated and a standard curve against concentrations of protein is constructed. A sample with an unknown protein concentration is electrophoresed with known concentration of BSA. The lane contained unknown sample is scanned and from the area the concentration of protein is determined.

B5(c) Gel Elution and Specific Activity

An aliquot of C-18 highly purified active fraction is subjected to SDS gel and sliced according to molecular weights described in FIG. 14. Proteins are eluted from the slices in 4M guanidine-HCl containing 0.5% Triton X-100, desalted, concentrated and assayed for endochondral bone forming activity as determined by calcium content. The C-18 highly active fractions and gel eluted substantially pure 30 kD osteogenic protein are implanted in varying concentrations in order to determine the half maximal bone inducing activity.

FIG. 14 demonstrates that the bone inducing activity is due to proteins eluted at 28–34 kD region. The recovery of activity after the gel elution step is determined by calcium content. FIGS. 20A and 20B represent the bone inducing activity for the various concentrations of 30 kD protein before and after gel elution as estimated by calcium content. The concentration of protein is determined by gel scanning in the 30 kD region. The data suggest that the half maximal activity for 30 kD protein before gel elution is 69 nanogram per 25 mg implant and is 21 nanogram per 25 mg implant after elution. Table 4 describes the yield, total specific activity, and fold purification of osteogenic protein at each step during purification. Approximately 500 ug of heparin sepharose I fraction, 130–150 ug of the HA ultrogel fraction, 10–12 ug of the gel filtration fraction, 4–5 ug of the heparin sepharose II fraction, 0.4–0.5 ug of the C-18 highly purified fraction, and 20–25 ng of gel eluted substantially purified is needed per 25 mg of implant for unequivocal bone formation for half maximal activity. Thus, 0.8–1.0 ng purified osteogenic protein per mg. of implant is required to exhibit half maximal bone differentiation activity in vivo.

TABLE 4

PURIFICATION OF BOP

Purification Steps	Protein (mg.)	Biological Activity Units*	Specific Activity Units/mg.	Purification Fold
Ethanol Precipitate**	30,000#	4,000	0.13	1
Heparin Sepharose I	1,200#	2,400	2.00	15
HA-Ultrogel	300#	2,307	7.69	59
Gel filtration	20#	1,600	80.00	615
Heparin Sepharose II	5#	1,000	200.00	1,538
C-18 HPLC	0.070@	150	2,043.00	15,715
Gel elution	0.004@	191	47,750.00	367,307

Values are calculated from 4 kg. of bovine bone matrix (800 g of demineralized matrix).
* One unit of bone forming activity is defined as the amount that exhibits half maximal bone differentiation activity compared to rat demineralized bone matrix, as determined by calcium content of the implant on day 12 in rats.
# Proteins were measured by absorbance at 280 nm.
@ Proteins were measured by gel scanning method compared to known standard protein, bovine serum albumin.
** Ethanol-precipitated guanidine extract of bovine bone is a weak inducer of bone in rats, possibly due to endogenous inhibitors. This precipitate is subjected to gel filtration and proteins less than 50 kD were separated and used for bioassay.

C. CHEMICAL CHARACTERIZATION OF BOP

C1. Molecular Weight and Structure

Electrophoresis of the most active fractions from reverse phase C-18 chromatography on non-reducing SDS polyacrylamide gels reveals a single band at about 30 kD as detected by both Coomassie blue staining (FIG. 3A) and autoradiography.

In order to extend the analysis of BOP, the protein was examined under reducing conditions. FIG. 3B shows an SDS gel of BOP in the presence of dithiothreitol. Upon reduction, 30 kD BOP yields two species which are stained with Coomassic blue dye: a 16 kD species and an 18 kD species. Reduction causes loss of biological activity. Methods for the efficient elution of the proteins from SDS gels have been tested, and a protocol has been developed to achieve purification of both proteins. The two reduced BOP species have been analyzed to determine if they are structurally related. Comparison of the amino acid composition of the two proteins (as disclosed below) shows little differences, indicating that the native protein may comprise two chains having some homology.

C2. Charge Determination

Isoelectric focusing studies are initiated to further evaluate the 30 kD protein for possibile heterogeneity. Results to date have not revealed any such heterogeneity. The oxidized and reduced species migrate as diffuse bands in the basic region of the isoelectric focusing gel,

using the iodinated 30 kD protein for detection. Using two dimensional gel electrophoresis and Con A for detection, the oxidized 30 kD protein show one species migrating in the same basic region as the iodinated 30 kD protein. The diffuse character of the band may be traced to the presence of carbohydrate attached to the protein.

C3. Presence of Carbohydrate

The 30 kD protein has been tested for the presence of carbohydrate by Concanavalin A (Con A) blotting after SDS-PAGE and transfer to nitrocellulose paper. The results demonstrate that the 30 kD protein has a high affinity for Con A, indicating that the protein is glycosylated (FIG. 4A). In addition, the Con A blots provide evidence for a substructure in the 30 kD region of the gel, suggesting heterogeneity due to varying degrees of glycosylation. After reduction (FIG. 4B), Con A blots show evidence for two major components at 16 kD and 18 kD. In addition, it has been demonstrated that no glycosylated material remains at the 30 kD region after reduction.

In order to confirm the presence of carbohydrate and to estimate the amount of carbohydrate attached, the 30 kD protein is treated with N-glycanase, a deglycosylating enzyme with a broad specificity. Samples of the ^{125}I-labelled 30 kD protein are incubated with the enzyme in the presence of SDS for 24 hours at 37° C. As observed by SDS-PAGE, the treated samples appear as a prominent species at about 27 kD (FIG. 5B-1). Upon reduction, the 27 kD species is reduced to species having a molecular weight of about 14 kD–16 kD (FIG. 5B-2).

Chemical cleavage of the carbohydrate moieties using hydrogen fluoride (HF) is performed to assess the role of carbohydrate on the bone inducing activity of BOP in vivo. Active osteogenic protein fractions pooled from the C-18 chromatography step are dried in vacuo over P_2O_5 in a polypropylene tube, and 50 ml freshly distilled anhydrous HF at −70° C. is added. After capping the tube tightly, the mixture is kept at 0° C. in an ice-bath with occasional agitation for 1 hr. The HF is then evaporated using a continuous stream of dry nitrogen gas. The tube is removed from the ice bath and the residue dried in vacuo over P_2O_5 and KOH pellets.

Following drying, the samples are dissolved in 100 ml of 50% acetonitrile/0.1% TFA and aliquoted for SDS gel analysis, Con A binding, and biological assay. Aliquots are dried and dissolved in either SDS gel sample buffer in preparation for SDS gel analysis and Con A blotting or 4M guanidine-HCl, 50 mM Tris-HCl, pH 7.0 for biological assay.

The results show that samples are completely deglycosylated by the HF treatment: Con A blots after SDS gel electrophoreses and transfer to Immobilon membrane showed no binding of Con A to the treated samples, while untreated controls were strongly positive at 30 kD. Coomassie gels of treated samples showed the presense of a 27 kD band instead of the 30 kD band present in the untreated controls.

C4. Chemical and Enzymatic Cleavage

Cleavage reactions with CNBr are analyzed using Con A binding for detection of fragments associated with carbohydrate. Cleavage reactions are conducted using trifluoroacetic acid (TFA) in the presence and absence of CNBr. Reactions are conducted at 37° C. for 18 hours, and the samples are vacuum dried. The samples are washed with water, dissolved in SDS gel sample buffer with reducing agent, boiled and applied to an SDS gel. After electrophoresis, the protein is transferred to Immobilon membrane and visualized by Con A binding. In low concentrations of acid (1%), CNBr cleaves the majority of 16 kD and 18 kD species to one product, a species about 14 kD. In reactions using 10% TFA, a 14 kD species is observed both with and without CNBr.

Four proteolytic enzymes are used in these experiments to examine the digestion products of the 30 kD protein: 1) V-8 protease; 2) Endo Lys C protease; 3) pepsin; and 4) trypsin. Except for pepsin, the digestion buffer for the enzymes is 0.1M ammonium bicarbonate, pH 8.3. The pepsin reactions are done in 0.1% TFA. The digestion volume is 100 ml and the ratio of enzyme to substrate is 1:10. ^{125}I-labelled 30 kD osteogenic protein is added for detection. After incubation at 37° C. for 16 hr., digestion mixtures are dried down and taken up in gel sample buffer containing dithiothreitol for SDS-PAGE. FIG. 6 shows an autoradiograph of an SDS gel of the digestion products. The results show that under these conditions, only trypsin digests the reduced 16 kD/18 kD species completely and yields a major species at around 12 kD. Pepsin digestion yields better defined, lower molecular weight species. However, the 16 kD/18 kD fragments were not digested completely. The V-8 digest shows limited digestion with one dominant species at 16 kD.

C5. Protein Sequencing

To obtain amino acid sequence data, the protein is cleaved with trypsin or Endoproteinase Asp-N (EndoAsp-N). The tryptic digest of reduced and carboxymethylated 30 kD protein (approximately 10 mg) is fractionated by reverse-phase HPLC using a C-8 narrowbore column (13 cm×2.1 mm ID) with a TFA/acetonitrile gradient and a flow rate of 150 ml/min. The gradient employs (A) 0.06% TFA in water and (B) 0.04% TFA in water and acetonitrile (1:4; v:v). The procedure was 10% B for five min., followed by a linear gradient for 70 min. to 80% B, followed by a linear gradient for 10 min. to 100% B. Fractions containing fragments as determined from the peaks in the HPLC profile (FIG. 7A) are rechromatographed at least once under the same conditions in order to isolate single components satisfactory for sequence analysis.

The HPLC profiles of the similarly digested 16 kD and 18 kD subunits are shown in FIGS. 7B and 7C, respectively. These peptide maps are similar suggesting that the subunits are identical or are closely related.

The 16 kD and 18 kD subunits are digested with Endo Asp N proteinase. The protein is treated with 0.5 mg EndoAsp-N in 50 mM sodium phosphate buffer, pH 7.8 at 36° C. for 20 hr. The conditions for fractionation are the same as those described previously for the 30 kD, 16 kD, and 18 kD digests. The profiles obtained are shown in FIGS. 16A and 16B.

Various of the peptide fragments produced using the foregoing procedures have been analyzed in an automated amino acid sequencer (Applied Biosystems 470A with 120A on-line PTH analysis). The following sequence data has been obtained:

(1) S-F-D-A-Y-Y-C-S-G-A-C-Q-F-P-M-P-K;
(2) S-L-K-P-S-N-Y-A-T-I-Q-S-I-V;
(3) A-C-C-V-P-T-E-L-S-A-I-S-M-L-Y-L-D-E-N-E-K;
(4) M-S-S-L-S-I-L-F-F-D-E-N-K;
(5) V-G-V-V-P-G-I-P-E-P-C-C-V-P-E;

(6) V-D-F-A-D-I-G;
(7) V-P-K-p-C-C-A-P-T;
(8) D-E-Q-T-L-K-K-A-R-R-K-Q-W-I-?-P;
(9) D-I-G-?-S-E-W-I-I-?-P;
(10) S-I-V-R-A-V-G-V-V-P-G-I-P-E-P-?-?-V;
(11) D-?-I-V-A-P-P-Q-Y-H-A-F-Y;
(12) D-E-N-K-N-V-V-L-K-V-Y-P-N-M-T-V-E;
(13) S-Q-T-L-Q-F-D-E-Q-T-L-K-?-A-R-?-K-Q;

C6. Amino Acid Analysis

Strategies for obtaining amino acid composition were developed using gel elution from 15% SDS gels, transfer onto Immobilon, and hydrolysis. Immobilon membrane is a polymer of vinylidene difluoride and, therefore, is not susceptible to acid cleavage. Samples of oxidized (30 kD) and reduced (16 kD and 18 kD) BOP are electrophoresed on a gel and transferred to Immobilon for hydrolysis and analysis as described below. The composition data generated by amino acid analyses of 30 kD BOP is reproducible, with some variation in the number of residues for a few amino acids, especially cysteine and isoleucine.

Samples are run on 15% SDS gels, transferred to Immobilon, and stained with Coomassie blue. The bands of interest are excised from the Immobilon, with a razor blade and placed in a 6×50 mm Corning test tube cleaned by pyrolysis at 550° C. When cysteine is to be determined, the samples are treated with performic acid, which converts cysteine to cysteic acid. Cysteic acid is stable during hydrolysis with HCl, and can be detected during the HPLC analysis by using a modification of the normal Pico-Tag eluents (Millipore) and gradient. The performic acid is made by mixing 50 ml 30% hydrogen peroxide with 950 ml 99% formic acid, and allowing this solution to stand at room temperature for 2 hr. The samples are then treated with performic acid (PFA); 20 ml PFA is pippetted onto each sample and placed in an ice bath at 4° C. for 2.5 hours. After 2.5 hr. the PFA is removed by drying in vacuo, and the samples are then hydrolyzed. A standard protein of known composition and concentration containing cysteine is treated with PFA and hydrolyzed concurrently with the osteogenic protein samples, to take as a control for hydrolysis and amino acid chromatography.

The hydrolysis of the osteogenic protein samples is done in vacuo. The samples, with empty tubes and Immobilon blanks, are placed in a hydrolysis vessel which is placed in a dry ice/ethanol bath to keep the HCl from prematurely evaporating. 200 ml 6N HCl containing 2% phenol and 0.1% stannous chloride are added to the hydrolysis vessel outside the tubes containing the samples. The hydrolysis vessel is then sealed, flushed with prepurified nitrogen, evacuated, and then held at 115° C. for 24 hours, after which time the HCl is removed by drying in vacuo.

After hydrolysis, each piece of Immobilon is transferred to a fresh tube, where it is rinsed twice with 100 ml 0.1% TFA, 50% acetonitrile. The washings are returned to the original sample tube, which is then redried as below. A similar treatment of amino acid analysis on Immobilon can be found in the literature (LeGendre and Matsudaira (1988) Biotechniques 6:154–159).

The samples are redried twice using 2:2:1 ethanol:water:triethylamine and allowed to dry at least 30 min. after each addition of redry reagent. These redrying steps bring the sample to the proper pH for derivatization.

The samples are derivatized using standard methodology. The solution is added to each sample tube. The tubes are placed in a desiccator which is partially evacuated, and are allowed to stand for 20 min. The desiccator is then fully evacuated, and the samples are dried for at least 3 hr. After this step the samples may be stored under vacuum at −20° C. or immediately diluted for HPLC. The samples are diluted with Pico-Tag Sample Diluent (generally 100 ml) and allowed to stand for 20 min., after which they are analyzed on HPLC using the Pico Tag chromatographic system with some minor changes involving gradients, eluents, initial buffer conditions and oven temperature.

After HPLC analysis, the compositions are calculated. The molecular weights are assumed to be 14.4 kD, 16.2 kD, and 27 kD to allow for 10% carbohydrate content. The number of residues is approximated by dividing the molecular weight by the average molecular weight per amino acid, which is 115. The total picomoles of amino acid recovered is divided by the number of residues, and then the picomoles recovered for each amino acid is divided by the number of picomoles per residue, determined above. This gives an approximate theoretical number of residues of each amino acid in the protein. Glycine content may be overestimated in this type of analysis.

Composition data obtained are shown in TABLE 5.

TABLE 5

	BOP Amino Acid Analyses		
Amino Acid	30 kD	16 kD	18 kD
Aspartic Acid/ Asparagine	22	14	15
Glutamic Acid/ Glutamine	24	14	16
Serine	24	16	23
Glycine	29	18	26
Histidine	5	*	4
Arginine	13	6	6
Threonine	11	6	7
Alanine	18	11	12
Proline	14	6	6
Tyrosine	11	3	3
Valine	14	8	7
Methionine	3	0	2
Cysteine**	16	14	12
Isoleucine	15	14	10
Leucine	15	8	9
Phenylalanine	7	4	4
Tryptophan	ND	ND	ND
Lysine	12	6	6

*This result is not integrated because histidine is present in low quantities.
**Cysteine is corrected by percent normally recovered from performic acid hydrolysis of the standard protein.

The results obtained from the 16 kD and 18 kD subunits, when combined, closely resemble the numbers obtained from the native 30 kD protein. The high figures obtained for glycine and serine are most likely the result of gel elution.

D. Purification of Human Osteogenic Protein

Human bone is obtained from the Bone Bank, (Massachusetts General Hospital, Boston, Mass.), and is milled, defatted, demarrowed and demineralized by the procedure disclosed above. 320 g of mineralized bone matrix yields 70–80 g of demineralized bone matrix. Dissociative extraction and ethanol precipitation of the matrix gives 12.5 g of guanidine-HCl extract.

One third of the ethanol precipitate (0.5 g) is used for gel filtration through 4M guanidine-HCl (FIG. 10A).

Approximately 70–80 g of ethanol precipitate per run is used. In vivo bone inducing activity is localized in the fractions containing proteins in the 30 kD range. They are pooled and equilibrated in 6M urea, 0.5M NaCl buffer, and applied directly onto a HAP column; the bound protein is eluted stepwise by using the same buffer containing 100 mM and 500 mM phosphate (FIG. 10B). Bioassay of HAP bound and unbound fractions demonstrates that only the fraction eluted by 100 mM phosphate has bone inducing activity in vivo. The biologically active fraction obtained from HAP chromatography is subjected to heparin-Sepharose affinity chromatography in buffer containing low salt; the bound proteins are eluted by 0.5M NaCl FIG. 10D. FIG. 10C describes the elution profile for the intervening gel filtration step described above). Assaying the heparin-Sepharose fractions shows that the bound fraction eluted by 0.5M NaCl have bone-inducing activity. The active fraction is then subjected to C-18 reverse phase chromatography.

The active fraction can then be subjected to SDS-PAGE as noted above to yield a band at about 30 kD comprising substantially pure human osteogenic protein.

E. Biosynthetic Probes For Isolation of Genes Encoding Native Osteogenic Protein

E-1. Probe Design

A synthetic consensus gene shown in FIG. 13 was designed as a hybridization probe (and to encode a consensus protein, see below) based on amino acid predictions from homology with the TGF-beta gene family and using human codon bias as found in human TGF-beta. The designed concensus sequence was then constructed using known techniques involving assembly of oligonucleotides manufactured in a DNA synthesizer.

Tryptic peptides derived from BOP and sequenced by Edman degradation provided amino acid sequences that showed strong homology with the Drosophila DPP protein sequence (as inferred from the gene), the Xenopous VG1 protein, and somewhat less homology to inhibin and TGF-beta, as demonstrated below in TABLE 6.

TABLE 6

protein	amino acid sequence	homology
(BOP)	SFDAYYCSGACQFPS	(9/15 matches)
(DPP)	GYDAYYCHGKCPFFL	
(BOP)	SFDAYYCSGACQFPS	(6/15 matches)
(Vgl)	GYMANYCYGECPYPL	
(BOP)	SFDAYYCSGACQFPS	(5/15 matches)
(inhibin)	GYHANYCEGECPSHI	
(BOP)	SFDAYYCSGACQFPS	(4/15 matches)
(TGF-beta)	GYHANFCLGPCPYIW	
(BOP)	K/RACCVPTELSAISMLYLDEN	(12/20 matches)
(Vgl)	LPCCVPTKMSPISMLFYDNN	
(BOP)	K/RACCVPTELSAISMLYLDEN	(12/20 matches)
(inhibin)	KSCCVPTKLRPMSMLYYDDG	

TABLE 6-continued

protein	amino acid sequence	homology
(BOP)	K/RACCVPTELSAISMLYLDE	(6/19 matches)
(TGF-beta)	APCCVPQALEPLPIVYYVG	
(BOP)	K/RACCVPTELSAISMLYLDEN	(12/20 matches)
(DPP)	KACCVPTQLDSVAMLYLNDQ	
(BOP)	LYVDF	(5/5 matches)
(DPP)	LYVDF	
(BOP)	LYVDF	(4/5 matches)
(Vgl)	LYVEF	
(BOP)	LYVDF	(4/5 matches)
(TGF-beta)	LYIDF	
(BOP)	LYVDF	(2/4 matches)
(inhibin)	FFVSF	

*-match

In determining the amino acid sequence of an osteogenic protein (from which the nucleic acid sequence can be determined), the following points were considered: (1) the amino acid sequence determined by Edman degradation of osteogenic protein tryptic fragments is ranked highest as long as it has a strong signal and shows homology or conservative changes when aligned with the other members of the gene family; (2) where the sequence matches for all four proteins, it is used in the synthetic gene sequence; (3) matching amino acids in DPP and Vgl are used; (4) If Vgl or DPP diverged but either one were matched by inhibin or by TGF-beta, this matched amino acid is chosen; (5) where all sequences diverged, the DPP sequence is initially chosen, with a later plan of creating the Vgl sequence by mutagenesis kept as a possibility. In addition, the consensus sequence is designed to preserve the disulfide crosslinking and the apparent structural homology.

One purpose of the originally designed synthetic consensus gene sequence, designated COP0, (see FIG. 13), was to serve as a probe to isolate natural genes. For this reason the DNA was designed using human codon bias. Alternatively, probes may be constructed using conventional techniques comprising a group of sequences of nucleotides which encode any portion of the amino acid sequence of the osteogenic protein produced in accordance with the foregoing isolation procedure. Use of such pools of probes also will enable isolation of a DNA encoding the intact protein.

E-2. Retrieval of Genes Encoding Osteogenic Protein from Genomic Library

A human genomic library (Maniatis-library) carried in lambda phage (Charon 4A) was screened using the COP0 consensus gene as probe. The initial screening was of 500,000 plaques (10 plates of 50,000 each). Areas giving hybridization signal were punched out from the plates, phage particles were eluted and plated again at a density of 2000–3000 plaques per plate. A second hybridization yielded plaques which were plated once more, this time at a density of ca 100 plaques per plate allowing isolation of pure clones. The probe (COP0) is a 300 base pair BamHI-PstI fragment restricted from an amplification plasmid which was labeled using alpha 32

dCTP according to the random priming method of Feinberg and Vogelstein, Anal. Biochem., 137, 266–267, 1984. Prehybridization was done for 1 hr in 5x SSPE, 10x Denhardt's mix, 0.5% SDS at 50° C. Hybridization was overnight in the same solution as above plus probe. The washing of nitrocellulose membranes was done, once cold for 5 min. in 1×SSPE with 0.1% SDS and twice at 50° C. for 2×30 min. in the same solution.

Using this procedure, twenty-four positive clones were found. Two of these yielded the genes corresponding to BMP-2b, one yielded BMP-3 (see PCT US 87/01537) and two contained a gene never before reported designated OP1, osteogenic protein-1 described below.

Southern blot analysis of lambda #13 DNA showed that an approximately 3 kb BamHI fragment hybridized to the probe. (See FIG. 1B). This fragment was isolated and subcloned into a bluescript vector (at the BamHI site). The clone was further analyzed by Southern blotting and hybridization to the COP0 probe. This showed that a 1 kb (approx.) EcoRI fragment strongly hybridized to the probe. This fragment was subcloned into the EcoRI site of a bluescript vector, and sequenced. Analysis of this sequence showed that the fragment encoded the carboxy terminus of a protein, named osteogenic protein-1 (OP1). The protein was identified by amino acid homology with the TGF-beta family. For this comparison cysteine patterns were used and then the adjacent amino acids were compared. Consensus splice signals were found where amino acid homologies ended, designating exon intron boundaries. Three exons were combined to obtain a functional TGF-beta-like domain containing seven cysteines. Two introns were deleted by looping out via primers bridging the exons using the single stranded mutagenesis method of Kunkel. Also, upstream of the first cysteine, an EcoRI site and an asp-pro junction for acid cleavage were introduced, and at the 3' end a PstI site was added by the same technique. Further sequence information (penultimate exon) was obtained by sequencing the entire insert. The sequencing was done by generating a set of unidirectionally deleted clones (Ozkaynak, E., and Putney, S.: Biotechniques, 5, 770-773, 1987). The obtained sequence covers about 80% of the TGF-beta-like region of OP1 and is set forth in FIG. 1A. The complete sequence of the TGF-beta like region was obtained by first subcloning all EcoRI generated fragments of lambda clone #13 DNA and sequencing a 4 kb fragment that includes the first portion of the TGF-beta like region (third exon counting from end) as well as sequences characterized earlier. The gene on an EcoRI to PstI fragment was inserted into an *E. coli* expression vector controlled by the trp promoter-operator to produce a modified trp LE fusion protein with an acid cleavage site. The OP1 gene encodes amino acids corresponding substantially to a peptide found in sequences of naturally sourced material. The amino acid sequence of what is believed to be its active region is set forth below:

```
                1       10                  20          30          40
OP1             LYVSFR--DLGWQDWIIAPEGYAAYYCEGECAFPLNS
                                  50          60          70
YMNATN---H--AIVQTLVHFINPET--VPKPCCAPTQLNA
                80          90          100
ISVLYFDDSSNVILKKYRNMVVRACGCH
```

A longer active sequence is:

```
                                                        -5
                                                        HQRQA
                1       10                  20          30          40
OP1             CKKHELYVSFR--DLGWQDWIIAPEGYAAYYCEGECAFPLNS
                                  50          60          70
YMNATN---H--AIVQTLVHFINPET--VPKPCCAPTQLNA
                80          90          100
ISVLYFDDSSNVILKKYRNMVVRACGCH
```

E-3. Probing cDNA Library

Another example of the use of pools of probes to enable isolation of a DNA encoding the intact protein is shown by the following. Cells known to express the protein are extracted to isolate total cytoplasmic RNA. An oligo-dT column can be used to isolate mRNA. This mRNA can be size fractionated by, for example, gel electrophoresis. The fraction which includes the mRNA of interest may be determined by inducing transient expression in a suitable host cell and testing for the presence of osteogenic protein using, for example, antibody raised against peptides derived from the tryptic fragments of osteogenic protein in an immunoassay. The mRNA fraction is then reverse transcribed to single stranded cDNA using reverse transcriptase; a second complementary DNA strand can then be synthesized using the cDNA as a template. The double-standard DNA is then ligated into vectors which are used to transfect bacteria to produce a cDNA library.

The radiolabelled consensus sequence, portions thereof, and/or synthetic deoxy oligonucleotides complementary to codons for the known amino acid sequences in the osteogenic protein may be used to identify which of the DNAs in the cDNA library encode the full length osteogenic protein by standard DNA-DNA hybridization techniques.

The cDNA may then be integrated in an expression vector and transfected into an appropriate host cell for protein expression. The host may be a prokaryotic or eucaryotic cell since the former's inability to glycosylate osteogenic protein will not effect the protein's enzymatic activity. Useful host cells include *Saccharomyces, E. coli,* and various mammalian cell cultures. The vector may additionally encode various signal sequences for protein secretion and/or may encode osteogenic protein as a fusion protein. After being translated, protein may be purified from the cells or recovered from the culture medium.

II. RECOMBINANT NON-NATIVE OSTEOGENIC PROTEIN CONSTRUCTS

A. Protein Design

This section discloses the production of novel recombinant proteins capable of inducing cartilage and endochondral bone comprising a protein structure duplicative of the functional domain of the amino acid sequence encoded by consensus DNA sequences derived from a family of natural proteins implicated in tissue development. These gene products/proteins are known to exist in active form as dimers and are, in general, processed from a precursor protein to produce an active C-terminal domain of the precursor.

The recombinant osteogenic/chondrogenic proteins are "novel" in the sense that, as far as applicants are aware, they do not exist in nature or, if they do exist, have never before been associated with bone or cartilage formation. The approach to design of these proteins was to employ amino acid sequences, found in the native isolates described above, in polypeptide structures which are patterned after certain proteins reported in the literature, or the amino acid sequences inferred from DNAs reported in the literature. Thus, using the design criteria set forth above in the probe design section, and refining the amino acid sequence as more protein sequence information was learned, a series of synthetic proteins were designed with the hope and intent that they might have osteogenic or chondrogenic activity when tested in the bioassay system disclosed below.

It was noted, for example, that DPP from drosophila, VG1 from Xenopus, the TGF beta family of proteins, and to a lesser extent, alpha and beta inhibins, had significant homologies with certain of the sequences derived from the naturally sourced OP product. (FIG. 18.) Study of these proteins led to the realization that a portion of the sequence of each had a structural similarity observable by analysis of the positional relationship of cysteines and other amino acids which have an important influence on three dimensional protein conformation. It was noted that a region of these sequences had a series of seven cysteines, placed very nearly in the same relative positions, and certain other amino acids in sequence as set forth below:

```
          10        20        30        40        50
CXXXXLXVXFXDXGWXXWXXXPXGXXAXYCXGXCXXPXXXXXXXXNHAXX

          60        70        80        90        100
QXXVXXXNXXXXPXXCCXPXXXXXXXXLXXXXXXXVXLXXYXXMXVXXCXCX
```

wherein each X independently represents an amino acid. Expression experiments with constructs patterned after this template amino acid sequence showed activity occurred with a shorter sequence having only six cysteines:

```
          10        20        30        40        50
LXVXFXDXGWXXWXXXPXGXXAXYCXGXCXXPXXXXXXXXNHAXX

          60        70        80        90        100
QXXVXXXNXXXXPXXCCXPXXXXXXXXLXXXXXXXVXLXXYXXMXVXXCXCX
```

wherein each X independently represents an amino acid. Within these generic structures are a multiplicity of specific sequences which have osteogenic or chondrogenic activity. Preferred structures are those having the amino acid sequence:

```
          10        20        30        40        50
CKRHPLYVDFRDVGWNDWI VAPPGYHAFYCHGECPFPL ADHL NS TNHAI V
 RRRS K S S L   QE  VI S E FD Y  E A AY MPES MKAS   VI
   KE F E K I   DN       L     N  S   Q  I TK F P    TL
    Q     A     S       K

          60        70        80        90        100
QTLVNS VNPGKI PKACCVPTEL S AI S MLYLDENENVVLKNYQDMVVEGCGCR
 SI  HAI  SEQV EP   A EQMNS LAI   FFNDQDK I  RK EE  T  DA H H
   RF     T   S       K DPV V   Y N S       H RN    RS
    N     S                     K         P      E
```

wherein, in each position where more than one amino acid is shown, any one of the amino acids shown may be used. Novel active proteins also are defined by amino acid sequences comprising an active domain beginning at residue number 6 of this sequence, i.e, omitting the N terminal CXXXX, or omitting any of the preferred specific combinations such as CKRHP, CRRKQ, CKRHE, etc, resulting in a construct having only 6 cysteine residues. After this work, PCT 87/01537 was published, and it was observed that the proteins there identified as BMPII a and b and BMPIII each comprised a region embodying this generic structure. These proteins were not demonstrated to be osteogenic in the published application. However, applicants discovered that a subpart of the amino acid sequence of these proteins, properly folded, and implanted as set forth herein, is active. These are disclosed herein as CBMPIIa, CBMPIIb, and CBMPIII. Also, the OP1 protein was observed to exhibit the same generic structure.

Thus, the preferred osteogenic proteins are expressed from recombinant DNA and comprise amino acid sequences including any of the following sequences:

```
Vg1   1         10        20        30        40
      CKKRHLYVEFK—DVGWQNWVIAPQGYMANYCYGECPYPLTE

                50        60        70
      ILNGSN---H—AILQTLVHSIEPED—IPLPCCVPTKMSP
```

-continued

```
                80              90              100
ISMLFYDNNDNVVLRHYENMAVDECGCR

DPP   1         10              20              30              40
CRRHSLYVDFS—DVGWDDWIVAPLGYDAYYCHGKCPFPLAD

                    50              60              70
HFNSTN···H—AVVQTLVNNNNPGK—VPKACCVPTQLDS

               80              90              100
VAMLYLNDQSTVVLKNYQEMTVVGCGCR

OP1   1       10              20              30              40
      LYVSFR—DLGWQDWIIAPEGYAAYYCEGECAFPLNS

                    50              60              70
YMNATN···H—AIVQTLVHFINPET—VPKPCCAPTQLNA

          80              90              100
ISVLYFDDSSNVILKKYRNMVVRACGCH

OP1                                                   −5
                                                      HQRQA
      1         10              20              30              40
CKKHELYVSFR—DLGWQDWIIAPEGYAAYYCEGECAFPLNS

                    50              60              70
YMNATN···H—AIVQTLVHFINPET—VPKPCCAPTQLNA

          80              90              100
ISVLYFDDSSNVILKKYRNMVVRACGCH

CBMP-2a 1         10              20              30              40
CKRHPLYVDFS—DVGWNDWIVAPPGYHAFYCHGECPFPLAD

                    50              60              70
HLNSTN···H—AIVQTLVNSVNS—K—IPKACCVPTELSA

               80              90              100
ISMLYLDENEKVVLKNYQDMVVEGCGCR

CBMP-2b 1         10              20              30              40
CRRHSLYVDFS—DVGWNDWIVAPPGYQAFYCHGDCPFPLAD

                    50              60              70
HLNSTN···H—AIVQTLVNSVNS—S—IPKACCVPTELSA

               80              90              100
ISMLYLDEYDKVVLKNYQEMVVEGCGCR

CBMP-3  1         10              20              30              40
CARRYLKVDFA—DIGWSEWIISPKSFDAYYCSGACQFPMPK

                    50              60              70
SLKPSN···H—ATIQSIVRAVGVVPGIPEPCCVPEKMSS

               80              90              100
LSILFFDENKNVVLKVYPNMTVESCARCR

COP1  1       10              20              30              40
      LYVDFQRDVGWDDWIIAPVDFDAYYCSGACQFPSAD

                    50              60              70
HFNSTN···H—AVVQTLVNNMNPGK—VPKPCCVPTELSA

               80              90              100
ISMLYLDENSTVVLKNYQEMTVVGCGCR

COP3  1       10              20              30              40
      LYVDFQRDVGWDDWIVAPPGYQAFYCSGACQFPSAD

                    50              60              70
HFNSTN···H—AVVQTLVNNMNPGK—VPKPCCVPTELSA

               80              90              100
ISMLYLDENEKVVLKNYQEMVVEGCGCR

COP4  1       10              20              30              40
      LYVDFS—DVGWDDWIVAPPGYQAFYCSGACQFPSAD

                    50              60              70
HFNSTN···H—AVVQTLVNNMNPGK—VPKPCCVPTELSA
```

-continued

```
          80          90          100
ISMLYLDENEKVVLKNYQEMVVEGCGCR

COP5  1       10          20          30          40
      LYVDFS—DVGWDDWIVAPPGYQAFYCHGECPFPLAD

                  50          60          70
HFNSTN---H—AVVQTLVNSVNSKI---PKACCVPTELSA

          80          90          100
ISMLYLDENEKVVLKNYQEMVVEGCGCR

COP7  1       10          20          30          40
      LYVDFS—DVGWNDWIVAPPGYHAFYCHGECPFPLAD

                  50          60          70
HLNSTN---H—AVVQTLVNSVNSKI---PKACCVPTELSA

          80          90          100
ISMLYLDENEKVVLKNYQEMVVEGCGCR

COP16                                 10
                                      PKHHSQRARKKNKN

      1       10          20          30          40
CRRHSLYVDFS—DVGWNDWIVAPPGYQAFYCHGECPFPLAD

                  50          60          70
HFNSTN---H—AVVQTLVNSVNSKI---PKAPCCVPTELSA

          80          90          100
ISMLYLDENEKVVLKNYQEMVVEGCGCR
```

As shown in FIG. 18, these sequences have considerable homology with the alpha and beta inhibins, three forms of TGF beta, and MIS.

B. Gene Prepartaion

The synthetic genes designed as described above preferably are produced by assembly of chemically synthesized oligonucleotides. 15–100 mer oligonucleotides may be synthesized on a Biosearch DNA Model 8600 Synthesizer, and purified by polyacrylamide gel electrophoresis (PAGE) in Tris-Borate-EDTA buffer (TBE). The DNA is then electroeluted from the gel. Overlapping oligomers may be phosphorylated by T4 polynucleotide kinase and ligated into larger blocks which may also be purifed by PAGE. Natural gene sequences and cDNAs also may be used for expression.

C. Expression

The genes can be expressed in appropriate prokaryotic hosts such as various strains of *E. coli.* For example, if the gene is to be expressed in *E. coli,* it must first be cloned into an expression vector. An expression vector (FIG. 21A) based on pBR322 and containing a synthetic trp promoter operator and the modified trp LE leader can be opened at the EcoRI and PSTI restriction sites, and a FB-FB COP gene fragment (FIG. 21B) can be inserted between these sites, where FB is fragment B of Staphylococcal Protein A. The expressed fusion protein results from attachment of the COP gene to a fragment encoding FB. The COP protein is joined to the leader protein via a hinge region having the sequence asp-pro-asn-gly. This hinge permits chemical cleavage of the fusion protein with dilute acid at the asp-pro site or cleavage at asn-gly with hydroxylamine, resulting in release of the COP protein.

D. Production of Active Proteins

The following procedure was followed for production of active recombinant protiens. *E. coli* cells containing the fusion proteins were lysed. The fusion proteins were purified by differential solubilization. In the case of the COP 1, 3, 4, 5, and 7 fusion proteins, cleavage was with dilute acid, and the resulting cleavage products were passed through a Sephacryl-200 HR column. The Sephacryl column separated most of the uncleaved fusion products from the COP 1, 3, 4, 5, and 7 analogs. In the case of the COP 16 fusion protein, cleavage was with a more concentrated acid, and an SP-Trisacryl column was used to separate COP 16, the leader protein, and the residual fusion protein. The COP fractions from any of the COP analogs were then subjected to HPLC on a semi-prep C-18 column. The HPLC column primarily separated the leader proteins and other minor impurities from the COP analogs.

Initial conditions for refolding of COP analogs were at pH 8.0 using Tris, GuHCl, dithiothreitol. Final conditions for refolding of COP analogs were at pH 8.0 using Tris, oxidized glutathione, and lower amounts of GuHCl and dithiothreitol.

E. Production of Antisera

Antisera to COP 7 and COP5 were produced in New Zealand white rabbits. Western blots demonstrate that the antisera react with COP 7 and COP5 preparations. Antisera to COP 7 has been tested for reactivity to bovine osteogenic protein samples. Western blots show a clear reaction with the 30 kD protein and, when reduced, with the 16 kD subunit. The immunoreactive species appears as a closely spaced doublet in the 16K subunit region, similar to the 16K doublet seen in Con A blots.

III. MATRIX PREPARATION

A. General Consideration of Matrix Properties

The carrier described in the bioassay section, infra, may be replaced by either a biodegradable-synthetic or synthetic-inorganic matrix (e.g., HAP, collagen, tricalcium phosphate, or polylactic acid, polyglycolic acid

and various copolymers thereof). Also xenogeneic bone may be used if pretreated as described below.

Studies have shown that surface charge, particle size, the presence of mineral, and the methodology for combining matrix and osteogenic protein all play a role in achieving successful bone induction. Perturbation of the charge by chemical modification abolishes the inductive response. Particle size influences the quantitative response of new bone; particles between 75 and 420 mm elicit the maximum response. Contamination of the matrix with bone mineral will inhibit bone formation. Most importantly, the procedures used to formulate osteogenic protein onto the matrix are extremely sensitive to the physical and chemical state of both the osteogenic protein and the matrix.

The sequential cellular reactions at the interface of the bone matrix/OF implants are complex. The multistep cascade includes: binding of fibrin and fibronectin to implanted matrix, chemotaxis of cells, proliferation of fibroblasts, differentiation into chondroblasts, cartilage formation, vascular invasion, bone formation, remodeling, and bone marrow differentiation.

A successful carrier for osteogenic protein must perform several important functions. It must bind osteogenic protein and act as a slow release delivery system, accommodate each step of the cellular response during bone development, and protect the osteogenic protein from nonspecific proteolysis. In addition, selected materials must be biocompatible in vivo and biodegradable; the carrier must act as a temporary scaffold until replaced completely by new bone. Polylactic acid (PLA), polyglycolic acid (PGA), and various combinations have different dissolution rates in vivo. In bones, the dissolution rates can vary according to whether the implant is placed in cortical or trabecular bone.

Matrix geometry, particle size, the presence of surface charge, and porosity or the presence of interstices among the particles of a size sufficient to permit cell infiltration, are all important to successful matrix performance. It is preferred to shape the matrix to the desired form of the new bone and to have dimensions which span non-union defects. Rat studies show that the new bone is formed essentially having the dimensions of the device implanted.

The matrix may comprise a shape-retaining solid made of loosely adhered particulate material, e.g., with collagen. It may also comprise a molded, porous solid, or simply an aggregation of close-packed particles held in place by surrounding tissue. Masticated muscle or other tissue may also be used. Large allogeneic bone implants can act as a carrier for the matrix if their marrow cavities are cleaned and packed with particles and the dispersed osteogenic protein.

B. Preparation of Biologically Active Allogenic Matrix

Demineralized bone matrix is prepared from the dehydrated diaphyseal shafts of rat femur and tibia as described herein to produce a bone particle size which pass through a 420 mm sieve. The bone particles are subjected to dissociative extraction with 4M guanidine-HCl. Such treatment results in a complete loss of the inherent ability of the bone matrix to induce endochondral bone differentiation. The remaining insoluble material is used to fabricate the matrix. The material is mostly collagenous in nature, and upon implantation, does not induce cartilage and bone. All new preparations are tested for mineral content and false positives before use. The total loss of biological activity of bone matrix is restored when an active osteoinductive protein fraction or a pure protein is reconstituted with the biologically inactive insoluble collagenous matrix. The osteoinductive protein can be obtained from any vertebrate, e.g., bovine, porcine, monkey, or human, or produced using recombinant DNA techniques.

C. Preparation of Deglycosylated Bone Matrix for Use in Xenogenic Implant

When osteogenic protein is reconstituted with collagenous bone matrix from other species and implanted in rat, no bone is formed. This suggests that while the osteogenic protein is xenogenic (not species specific), while the matrix is species specific and cannot be implanted cross species perhaps due to intrinsic immunogenic or inhibitory components. Thus, heretofore, for bone-based matrices, in order for the osteogenic protein to exhibit its full bone inducing activity, a species specific collagenous bone matrix was required.

The major component of all bone matrices is Type I collagen. In addition to collagen, extracted bone includes non-collagenous proteins which may account for 5% of its mass. Many non-collagenous components of bone matrix are glycoproteins. Although the biological significance of the glycoproteins in bone formation is not known, they may present themselves as potent antigens by virtue of their carbohydrate content and may constitute immunogenic and/or inhibitory components that are present in xenogenic matrix.

It has now been discovered that a collagenous bone matrix may be used as a carrier to effect bone inducing activity in xenogenic implants, if one first removes the immonogenic and inhibitory components from the matrix. The matrix is deglycosglated chemically using, for example, hydrogen fluoride to achieve this purpose.

Bovine bone residue prepared as described above is sieved, and particles of the 74–420 mM are collected. The sample is dried in vacuo over P_2O_5, transferred to the reaction vessel and anhydrous hydrogen fluoride (HF) (10–20 ml/g of matrix) is then distilled onto the sample at −70° C. The vessel is allowed to warm to 0° and the reaction mixture is stirred at this temperature for 60 min. After evaporation of the HF in vacuo, the residue is dried thoroughly in vacuo over KOH pellets to remove any remaining traces of acid.

Extent of deglycosylation can be determined from carbohydrate analysis of matrix samples taken before and after treatment with HF, after washing the samples appropriately to remove non-covalently bound carbohydrates.

The deglycosylated bone matrix is next treated as set forth below:

1) suspend in TBS (Tris-buffered Saline) 1 g/200 ml and stir at 4° C. for 2 hrs;
2) centrifuge then treated again with TBS, 1 g/200 ml and stir at 4° C. overnight; and
3) centrifuged; discard supernatant; water wash residue; and then lyophilized.

IV. FABRICATION OF DEVICE

Fabrication of osteogenic devices using any of the matrices set forth above with any of the osteogenic proteins described above may be performed as follows.

A. Ethanol Precipitation

In this procedure, matrix was added to osteogenic protein in guanidine-HCl. Samples were vortexed and incubated at a low temperature. Samples were then

further vortexed. Cold absolute ethanol was added to the mixture which was then stirred and incubated. After centrifugation (microfuge high speed) the supernatant was discarded. The reconstituted matrix was washed with cold concentrated ethanol in water and then lyophilized.

B. Acetonitrile Trifluoroacetic Acid Lyophilization

In this procedure, osteogenic protein in an acetonitrile trifluroacetic acid (ACN/TFA) solution was added to the carrier. Samples were vigorously vortexed many times and then lyophilized. Osteogenic protein was added in varying concentrations obtained at several levels of purity that have been tested to determine the most effective dose/purity level in rat in vivo assay.

C. Urea Lyophilization

For those proteins that are prepared in urea buffer, the protein is mixed with the matrix, vortexed many times, and then lyophilized. The lyophilized material may be used "as is" for implants.

V. IN VIVO RAT BIOASSAY

Substantially pure BOP, BOP-rich extracts comprising protein having the properties set forth above, and several of the synthetic proteins have been incorporated in matrices to produce osteogenic devices, and assayed in rat for endochondral bone. Studies in rats show the osteogenic effect to be dependent on the dose of osteogenic protein dispersed in the osteogenic device. No activity is observed if the matrix is implanted alone. The following sets forth guidelines for how the osteogenic devices disclosed herein might be assayed for determining active fractions of osteogenic protein when employing the isolation procedure of the invention, and evaluating protein constructs and matrices for biological activity.

A. Subcutaneous Implantation

The bioassay for bone induction as described by Sampath and Reddi (Proc. Natl. Acad. Sci. USA (1983) 80: 6591-6595), herein incorporated by reference, is used to monitor the purification protocols for endochondral bone differentiation activity. This assay consists of implanting the test samples in subcutaneous sites in allogeneic recipient rats under ether anesthesia. Male Long-Evans rats, aged 28-32 days, were used. A vertical incision (1 cm) is made under sterile conditions in the skin over the thoraic region, and a pocket is prepared by blunt dissection. Approximately 25 mg of the test sample is implanted deep into the pocket and the incision is closed with a metallic skin clip. The day of implantation is designated as day of the experiment. Implants were removed on day 12. The heterotropic site allows for the study of bone induction without the possible ambiguities resulting from the use of orthotopic sites.

B. Cellular Events

The implant model in rats exhibits a controlled progression through the stages of matrix induced endochondral bone development including: (1) transient infiltration by polymorphonuclear leukocytes on day one; (2) mesenchymal cell migration and proliferation on days two and three; (3) chondrocyte appearance on days five and six; (4) cartilage matrix formation on day seven; (5) cartiliage calcification on day eight; (6) vascular invasion, appearance of osteoblasts, and formation of new bone on days nine and ten; (7) appearance of osteoblastic and bone remodeling and dissolution of the implanted matrix on days twelve to eighteen; and (8) hematopoietic bone marrow differentiation in the ossicle on day twenty-one. The results show that the shape of the new bone conforms to the shape of the implanted matrix.

C. Histological Evaluation

Histological sectioning and staining is preferred to determine the extent of osteogenesis in the implants. Implants are fixed in Bouins Solution, embedded in parafilm, cut into 6-8 mm sections. Staining with toluidine blue or hemotoxylin/eosin demonstrates clearly the ultimate development of endochondrial bone. Twelve day implants are usually sufficient to determine whether the implants show bone inducing activity.

D. Biological Markers

Alkaline phosphatase activity may be used as a marker for osteogenesis. The enzyme activity may be determined spectrophotometrically after homogenization of the implant. The activity peaks at 9-10 days in vivo and thereafter slowly declines. Implants showing no bone development by histology should have little or no alkaline phosphatase activity under these assay conditions. The assay is useful for quantitation and obtaining an estimate of bone formation very quickly after the implants are removed from the rat. In order to estimate the amount of bone formation, the calcium content of the implant is determined.

Implants containing osteogenic protein at several levels of purity have been tested to determine the most effective dose/purity level, in order to seek a formulation which could be produced on an industrial scale. The results are as measured by specific acivity of alkaline phosphatase calcium content, and histological examination. The specific activity of alkaline phosphatase is elevated during onset of bone formation and then declines. On the other hand, calcium content is directly proportional to the total amount of bone that is formed. The osteogenic activity due to osteogenic protein is represented by "bone forming units". For example, one bone forming unit represents the amount of protein that is needed for half maximal bone forming activity as compared to rat demineralized bone matrix as control and determined by calcium content of the implant on day 12.

E. Results

E-1. Natural Sourced Osteogenic Protein

Dose curves are constructed for bone inducing activity in vivo at each step of the purification scheme by assaying various concentrations of protein. FIG. 11 shows representative dose curves in rats as determined by alkaline phosphatase. Similar results are obtained when represented as bone forming units. Approximately 10-12 mg of the Sephacryl-fraction, 3-4 mg of heparin-Sepharose-II fraction, 0.4-0.5 mg of the C-18 column purified fraction, and 20-25 ng of gel eluted highly purified 30 kD protein is needed for unequivocal bone formation (half maximum activity). 20-25 ng per 25 mg of implant is normally sufficient to produce endochondral bone. Thus, 1-2 ng osteogenic protein per mg of implant is a reasonable dosage, although higher dosages may be used. (See section IB5 on specific activity of osteogenic protein.)

E-2. Xenogenic Matrix Results

Deglycosylated xenogenic collagenous bone matrix (example: bovine) has been used instead of allogenic collagenous matrix to prepare osteogenic devices (see previous section) and bioassayed in rat for bone inducing activity in vivo. The results demonstrate that xenogenic collagenous bone matrix after chemical deglycosylation induces successful endochondral bone formation (FIG. 19). As shown by specific activity of alkaline phosphatase, it is evident that the deglycosylated xenogenic matrix induced bone whereas untreated bovine matrix did not.

Histological evaluation of implants suggests that the deglycosylated bovine matrix not only has induced bone in a way comparable to the rat residue matrix but also has advanced the developmental stages that are involved in endochondral bone differentiation. Compared to rat residue as control, the HF treated bovine matrix contains extensively remodeled bone. Ossicles are formed that are already filled with bone marrow elements by 12 days. This profound action as elicited by deglycosylated bovine matrix in supporting bone induction is reproducible and is dose dependent with varying concentration of osteogenic protein.

E-3. Synthetic/Recombinant Proteins (COP5 , COP7)

The device that contained only rat carrier showed complete absence of new bone formation. The implant consists of carrier rat matrix and surrounding mesenchymal cells. Again, the devices that contained rat carrier and not correctly folded (or biologically inactive) recombinant protein also showed complete absence of bone formation. These implants are scored as cartilage formation (−) and bone formation (−). The endochondral bone formation activity is scored as zero percent (0%). (FIG. 22A)

Implants that included biologically active recombinant protein, however, showed evidence of endochondral bone formation. Histologically they showed new cartilage and bone formation.

The cartilage formation is scored as (+) by the presence of metachromatically stained chondrocytes in center of the implant, as (++) by the presence of numerous chondrocytes in many areas of the implant and as (+++) by the presence of abundant chondrocytes forming cartilage matrix and the appearance of hypertrophied chondrocytes accompanying cartilage calcification (FIG. 22B).

The bone formation is scored as (+) by the presence of osteoblast surrounding vascular endothelium forming new matrix, and as (++) by the formation of bone due to osteoblasts (as indicated by arrows) and further bone remodeling by the appearance of osteoblasts in apposition to the rat carrier. Vascular invasion is evident in these implants (FIG. 22B).

The overall bone inducing activity due to recombinant protein is represented as percent response of endochondral bone formation (see Table 7 below). The percent response means the area of the implant that is covered by newly induced cartilage and bone as shown by histology in low magnification.

TABLE 7

HISTOLOGICAL EVALUATION OF RECOMBINANT BONE INDUCTIVE PROTEINS

Implanted Protein	Cartilage Formation	Bone Formation	Percent Response in the Implant
COP-5	+++	++	15%
COP-5	++	+	5%
COP-7	+++	++	30%
COP-7	+++	++	20%
COP-7	++	+	20%
COP-7	++	+	10%
COP-7	+++	++	30%
COP-7	++	++	20%
COP-5	+++	++	20%

VI. ANIMAL EFFICACY STUDIES

Substantially pure osteogenic protein from bovine bone (BOP), BOP-rich osteogenic fractions having the properties set forth above, and several of the synthetic/-recombinant proteins have been incorporated in matrices to produce osteogenic devices. The efficacy of bone-inducing potential of these devices was tested in cat and rabbit models, and found to be potent inducers of osteogenesis, ultimately resulting in formation of mineralized bone. The following sets forth guidelines as to how the osteogenic devices disclosed herein might be used in a clinical setting.

A. Feline Model

The purpose of this study is to establish a large animal efficacy model for the testing of the osteogenic devices of the invention, and to characterize repair of massive bone defects and simulated fracture non-union encountered frequently in the practice of orthopedic surgery. The study is designed to evaluate whether implants of osteogenic protein with a carrier can enhance the regeneration of bone following injury and major reconstructive surgery by use of this large mammal model. The first step in this study design consists of the surgical preparation of a femoral osteotomy defect which, without further intervention, would consistently progress to non-union of the simulated fracture defect. The effects of implants of osteogenic devices into the created bone defects were evaluated by the following study protocol.

A-1. Procedure

Sixteen adult cats weighing less than 10 lbs. undergo unilateral preparation of a 1 cm bone defect in the right femur through a lateral surgical approach. In other experiments, a 2 cm bone defect was created. The femur is immediately internally fixed by lateral placement of an 8-hole plate to preserve the exact dimensions of the defect. There are three different types of materials implanted in the surgically created cat femoral defects: group I (n=3) is a control group which undergo the same plate fixation with implants of 4M guanidine-HCl-treated (inactivated) cat demineralized bone matrix powder (GuHCl-DBM) (360 mg); group II (n =3) is a positive control group implanted with biologically active demineralized bone matrix powder (DBM) (360 mg); and group III (n=10) undergo a procedure identical to groups I-II, with the addition of osteogenic protein onto each of the GuHCl-DBM carrier samples. To summarize, the group III osteogenic protein-treated animals are implanted with exactly the same material as the group I animals, but with the singular addition of osteogenic protein.

All animals are allowed to ambulate ad libitum within their cages post-operatively. All cats are injected with tetracycline (25 mg/kg SQ each week for four weeks) for bone labelling. All but four group III animals are sacrificed four months after femoral osteotomy.

A-2. Radiomorphometrics

In vivo radiomorphometric studies are carried out immediately post-op at 4, 8, 12 and 16 weeks by taking a standardized x-ray of the lightly anesthesized animal positioned in a cushioned x-ray jig designed to consistently produce a true anterio-posterior view of the femur and the osteotomy site. All x-rays are taken in exactly the same fashion and in exactly the same position on each animal. Bone repair is calculated as a function of mineralization by means of random point analysis. A final specimen radiographic study of the excised bone is taken in two planes after sacrifice. X-ray results are shown in FIG. 12, and displaced as percent of bone defect repair. To summarize, at 16 weeks, 60% of the group III femors are united with average 86% bone defect regeneration. By contrast, the group I GuHCl-DMB negative-control implants exhibit no bone growth at four weeks, less than 10% at eight and 12 weeks, and 16% (±10%) at 16 weeks with one of the five exhibiting a small amount of bridging bone. The group II DMB positive-control implants exhibited 18% (±3%) repair at four weeks, 35% at eight weeks, 50% (±10%) at twelve weeks and 70% (±12%) by 16 weeks, a statistical difference of $p<0.01$ compared to osteogenic protein at every month. One of the three (33%) is united at 16 weeks.

A-3. Biomechanics

Excised test and normal femurs are immediately studied by bone densitometry, wrapped in two layers of saline-soaked towels, placed in two sealed plastic bags, and stored at −20° C. until further study. Bone repair strength, load to failure, and work to failure are tested by loading to failure on a specially designed steel 4-point bending jig attached to an Instron testing machine to quantitate bone strength, stiffness, energy absorbed and deformation to failure. The study of test femurs and normal femurs yield the bone strength (load) in pounds and work to failure in joules. Normal femurs exhibit a strength of 96 (±12) pounds. osteogenic protein-implanted femurs exhibited 35 (±4) pounds, but when corrected for surface area at the site of fracture (due to the "hourglass" shape of the bone defect repair) this correlated closely with normal bone strength. Only one demineralized bone specimen was available for testing with a strength of 25 pounds, but, again, the strength correlated closely with normal bone when corrected for fracture surface area.

A-4. Histomorphometry/Histology

Following biomechanical testing the bones are immediately sliced into two longitudinal sections a the defect site, weighed, and the volume measured. One-half is fixed for standard calcified bone histomorphometrics with fluorescent stain incorporation evaluation, and one-half is fixed for decalcified hemotoxylin/eosin stain histology preparation.

A-5. Biochemistry

Selected specimens from the bone repair site ($n=6$) are homogenized in cold 0.15M NaCl, 3 mM $NaHCO_3$, pH 9.0 by a Spex freezer mill. The alkaline phosphatase activity of the supernatant and total calcium content of the acid soluble fraction of sediment are then determined.

A-6. Histopathology

The final autopsy reports reveal no unusual or pathologic findings noted at necropsy of any of the animals studied. Portion of all major organs are preserved for further study. A histopathological evaluation is performed on samples of the following organs: heart, lung, liver, both kidneys, spleen, both adrenals, lymph nodes, left and right quadriceps muscles at mid-femur (adjacent to defect site in experimental femur). No unusual or pathological lesions are seen in any of the tissues. Mild lesions seen in the quadriceps muscles are compatible with healing responses to the surgical manipulation at the defect site. Pulmonary edema is attributable to the euthanasia procedure. There is no evidence of any general systemic effects or any effects on the specific organs examined.

A-7. Feline Study Summary

The 1 cm and 2 cm femoral defect cat studies demonstrate that devices comprising a matrix containing disposed osteogenic protein can: (1) repair a weight-bearing bone defect in a large animal; (2) consistently induces bone formation shortly following (less than two weeks) implantation; and (3) induce bone by endochondral ossification, with a strength equal to normal bone, on a volume for volume basis. Furthermore, all animals remained healthy during the study and showed no evidence of clinical or histological laboratory reaction to the implanted device. In this bone defect model, there was little or no healing at control bone implant sites. The results provide evidence for the successful use of osteogenic devices to repair large, non-union bone defects.

B. Rabbit Model

B1. Procedure and Results

Eight mature (less than 10 lbs) New Zealand White rabbits with epiphyseal closure documented by X-ray were studied. The purpose of this study is to establish a model in which there is minimal or no bone growth in the control animals, so that when bone induction is tested, only a strongly inductive substance will yield a positive result. Defects of 1.5 cm are created in the rabbits, with implantation of: osteogenic protein ($n=5$), DBM ($n=8$), GuHCl-DBM ($n=6$), and no implant ($n=10$). Six osteogenic protein implants are supplied and all control defects have no implant placed.

Of the eight animals (one animal each was sacrificed at one and two weeks), 11 ulnae defects are followed for the full course of the eight week study. In all cases ($n=7$) following osteo-periosteal bone resection, the no implant animals establish no radiographic union by eight weeks. All no implant animals develop a thin "shell" of bone growing from surrounding bone present at four weeks and, to a slightly greater degree, by eight weeks. In all cases ($n=4$), radiographic union with marked bone induction is established in the osteogenic protein-implanted animals by eight weeks. As opposed to the no implant repairs, this bone repair is in the site of the removed bone.

Radiomorphometric analysis reveal 90% osteogenic protein-implant bone repair and 18% no-implant bone repair at sacrifice at eight weeks. At autopsy, the osteo-

genic protein bone appears normal, while "no implant" bone sites have only a soft fibrous tissue with no evidence of cartilage or bone repair in the defect site.

B-2. Allograft Device

In another experiment, the marrow cavity of the 1.5 cm ulnar defect is packed with activated osteogenic protein rabbit bone powder and the bones are allografted in an intercalary fashion. The two control ulnae are not healed by eight weeks and reveal the classic "ivory" appearance. In distinct contrast, the osteogenic protein-treated implants "disappear" radiographically by four weeks with the start of remineralization by six to eight weeks. These allografts heal at each end with mild proliferative bone formation by eight weeks.

This type of device serves to accelerate allograph repair.

B-3. Summary

These studies of 1.5 cm osteo-periosteal defects in the ulnae of mature rabbits show that: (1) it is a suitable model for the study of bone growth; (2) "no implant" or GuHCl negative control implants yield a small amount of periosteal-type bone, but not medullary or cortical bone growth; (3) osteogenic protein-implanted rabbits exhibited proliferative bone growth in a fashion highly different from the control groups; (4) initial studies show that the bones exhibit 50% of normal bone strength (100% of normal correlated vol:vol) at only eight weeks after creation of the surgical defect; and (5) osteogenic protein-allograft studies reveal a marked effect upon both the allograft and bone healing.

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The present embodiments are therefore to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

What is claimed is:

1. A protein, produced by expression of recombinant DNA in a host cell and isolated from said host cell, comprising a pair of polypeptide chains disulfide bonded to form a dimeric species, each of said pair of polypeptide chains having less than 200 amino acids in a sequence sufficiently duplicative of the sequence of:

```
COP5
1          10          20          30
      LYVDFSDVGWDDWIVAPPGYQAFYCHGECPFPL
40         50          60          70
ADHFNSTNHAVVQTLVNSVNSKIPKACCVPTELSA
     80          90          100
ISMLYLDENEKVVLKNYQEMVVEGCGCR

COP7
1          10          20          30
      LYVDFSDVGWNDWIVAPPGYHAFYCHGECPFPL
40         50          60          70
ADHLNSTNHAVVQTLVNSVNSKIPKACCVPTELSA
     80          90          100
ISMLYLDENEKVVLKNYQEMVVEGCGCR
```

such that said dimeric species has a conformation capable of inducing bone and cartilage formation when implanted in a mammal in association with a matrix.

2. The osteogenic protein of claim 1 having a molecular weight of about 30 kD when oxidized as determined by comparison to molecular weight standards in SDS-polyacrylamide gel.

3. The osteogenic protein of claim 2 further characterized by being glycosylated.

4. The osteogenic protein of claim 1 having a molecular weight of about 27 kD as determined by comparison to molecular weight standards in SDS-polyacrylamide gel electrophoresis.

5. The protein of claim 1 or 4 further characterized by being unglycosylated.

6. The protein of claim 1 comprising the amino acid sequences:

```
          10          20          30          40          50
CXXXXLXVXFXDXGWXXWXXXPXGXXAXYCXGXCXXPXXXXXXXXXNHAXX

          60          70          80          90          100
QXXVXXXNXXXXPXXCCXPXXXXXXXXLXXXXXXXVXLXXYXXMXVXXCXCX
```

wherein each X independently represents an amino acid.

7. The protein of claim 1 comprising the amino acid sequences:

```
          10          20          30          40          50
LXVXFXDXGWXXWXXXPXGXXAXYCXGXCXXPXXXXXXXXXNHAXX

          60          70          80          90          100
QXXVXXXNXXXXPXXCCXPXXXXXXXXLXXXXXXXVXLXXYXXMXVXXCXCX
```

wherein each X independently represents an amino acid.

8. The protein of claim 1 comprising the amino acid sequence:

```
OP1       1          10          20          30          40
          LYVSFR—DLGWQDWIIAPEGYAAYYCEGECAFPLNS

                     50          60          70
YMNATN ---H—AIVQTLVHFINPET—VPKPCCAPTQLNA

          80          90          100
ISVLYFDDSSNVILKKYRNMVVRACGCH.
```

9. The protein of claim 1 comprising the amino acid sequences:

```
                                                    -5
                                                   HQRQA
          1         10                  20        30        40
OP1       CKKHELYVSFR--DLGWQDWIIAPEGYAAYYCEGECAFPLNS

                          50          60          70
          YMNATN---H--AIVQTLVHFINPET--VPKPCCAPTQLNA

              80          90          100
          ISVLYFDDSSNVILKKYRNMVVRACGCH.
```

10. The protein of claim 1 comprising the product of expression of a DNA in a procaryotic host cell.

11. The protein of claim 1 produced by expression in a mammalian cell line.

12. The protein of claim 1 having a half-maximum bone forming activity of at least about 20–25 ng per 25 mg of implant.

13. The protein of claim 1 comprising the amino acid sequences:

$$CX_1RX_2X_3LX_4VX_5FX_6DX_7GWX_8X_9WX_{10}X_{11}X_{12}PX_{13}GX_{14}X_{15}AX_{16}YC$$

$$X_{17}GX_{18}CX_{19}X_{20}PX_{21}X_{22}X_{23}X_{24}X_{25}X_{26}X_{27}X_{28}NHAX_{29}X_{30}QX_{31}$$

$$X_{32}VX_{33}X_{34}X_{35}NX_{36}X_{37}X_{38}X_{39}PX_{40}X_{41}CCX_{42}PX_{43}X_{44}X_{45}X_{46}$$

$$X_{47}X_{48}X_{49}X_{50}LX_{51}X_{52}X_{53}X_{54}X_{55}X_{56}X_{57}VX_{58}LX_{59}X_{60}YX_{61}X_{62}M$$

$$X_{63}VX_{64}X_{65}CX_{66}CX_{67},$$

wherein X_1=(K or R); X_2=(H,R, or K); X_3=(P, S, E or Q); X_4=(Y, K or F); X_5=(D,S or E); X_6=(R, S, K or A); X_7=(V, L, or I); X_8=(N, Q, D or S); X_9=(D, E or N); X_{10}=(I or V); X_{11}=(I or V); X_{12}=(A or S); X_{13}=(P, E, L or K); X_{14}=(Y or F); X_{15}=(H or D); X_{16}=(F, Y or N); X_{17}=(H, E or S); X_{18}=(E or A); X_{19}=(P, A or Q); X_{20}=(F or Y); X_{21}=(L, M or I); X_{22}=(A, P or T); X_{22}=(D, E or K); X_{24}=(H or S); X_{25}=(L, M or F); X_{26}=(N or K); X_{27}=(S, A or P); X_{28}=(T or S); X_{29}=(I, V or T); X_{30}=(S, A, F or N); X_{35}=(V or I); X_{36}=(P or S); X_{37}=(G or E); X_{38}=(K, Q, T or S); X_{39}=(I or V); X_{40}=(K or E); X_{41}=(A, P or S); X_{42}=(V or A); X_{43}=(T or E); X_{44}=(E, Q or K); X_{45}=(L or M); X_{46}=(S N or D); X_{47}=(A, S or P); X_{48}=(I, L or V); X_{49}=(S or A); X_{50}=(M, I or V); X_{51}=(Y or F); X_{52}=(L, F or Y); X_{53}=(D or N); X_{54}=(E, D or N); X_{55}=(N or Q); X^{56}=(E, D, S or K); X_{57}=(N or K); X_{58}=(V or I); X_{59}=(K or R); X_{60}=(N, K or H); X_{61}=(Q, E, R or P); X_{62}=(D, E or N); X_{63}=(V or T); X_{64}=(E, D or R); X_{65}=(G, A, S or E); X_{66}=(G or H); and X_{67}=(R or H).

14. The protein of claim 1 comprising the amino acid sequences:

$$LX_1VX_2FX_3DX_4GWX_5X_6WX_7X_8X_9PX_{10}GX_{11}X_{12}AX_{13}YC$$

$$X_{14}GX_{15}CX_{16}X_{17}PX_{18}X_{19}X_{20}X_{21}X_{22}X_{23}X_{24}X_{25}NHAX_{26}X_{27}QX_{28}$$

-continued

$$X_{29}VX_{30}X_{31}X_{32}NX_{33}X_{34}X_{35}X_{36}PX_{37}X_{38}CCX_{39}PX_{40}X_{41}X_{42}X_{43}$$

$$X_{44}X_{45}X_{46}X_{47}LX_{48}X_{49}X_{50}X_{51}X_{52}X_{53}X_{54}VX_{55}LX_{56}X_{57}YX_{58}X_{59}M$$

$$X_{60}VX_{61}X_{62}CX_{63}CX_{64},$$

wherein X_1=(Y, K or F); X_2=(D, S or E); X_3=(R, S, K or A); X_4=(V, L, or I); X_5=(N, Q, D or S); X_6=(D, E or N); X_7=(I or V); X_8=(I or V); X_9=(A or S); X_{10}=(P, E, L or K); X_{11}=(Y or F); X_{12}=(H or D); X_{13}=(F, Y or N); X_{14}=(H, E or S); S_{15}=(E or A); X_{16}=(P, A or Q); X_{17}=(F or Y); X_{18}=(L, M or I); X_{19}=(A, P or T); X_{20}=(D, E or K); X_{21}=(H or S); X_{22}=(L, M or F); X_{23}=(N or K); X_{24}=(S, A or P); X_{25}=(T or S); X_{26}=(I, V, or T); X_{27}=(V, I or L); X_{28}=(T or S); X_{29}=(L or I); X_{30}=(N, H or R); X_{31}=(S, A, F or N); X_{32}=(V or I); X_{33}=(P or S); X_{34}=(G or E); X_{35}=(K, Q, T or S); X_{36}=(I or V); X_{37}=(K or E); X_{38}=(A, P or S); X_{39}=(V or A); X_{40}=(T or E); X_{41}=(E, Q or K); X_{42}=(L or M); X_{43}=(S N or D); X_{44}=(A, S or P); X_{45}=(I, L or V); X_{46}=(S or A); X_{47}=(M, I or V); X_{48}=(Y or F); X_{49}=(L, F or Y); X_{50}=(D or N); X_{51}=(E, D or N); X_{52}=(N or Q); X^{53}=(E, D, S or K); X_{54}=(N or K); X_{55}=(V or I); X_{56}=(K or R); X_{57}=(N, K or H); X_{58}=(Q, E, R or P); X_{59}=(D, E or N); X_{60}=(V or T); X_{61}=(E, D or R); X_{62}=(G, A, S or E); X_{63}=(G or H); and X_{64}=(R or H).

15. The protein of claim 1 having a half maximum bone-forming activity of at least 20–25 ng per 25 mg of matrix.

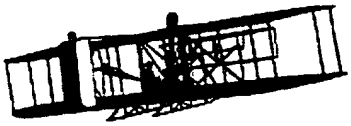
16. An isolated protein comprising a pair of polypeptide chains disulfide bonded to form a dimeric species, at least one of said polypeptide chains comprising the amino acid sequence:

```
          1        10        20        30        40
OP1       LYVSFRDLGWQDWIIAPEGYAAYYCEGECAFPLNS
                  50        60        70
          YMNATNHAIVQTLVHFINPETVPKPCCAPTQLNA
               80        90        100
          ISVLYFDDSSNVILKKYRNMVVRACGCH.
```

17. An isolated protein comprising a pair of polypeptide chains disulfide bonded to form a dimeric species, at least one of said polypeptide chains comprising the amino acid sequence:

```
                                                  -5
                                                  HQRQA
          1        10        20        30        40
OP1       CKKHELYVSFRDLGWQDWIIAPEGYAAYYCEGECAFPLNS
                  50        60        70
          YMNATNHAIVQTLVHFINPETVPKPCCAPTQLNA
               80        90        100
          ISVLYFDDSSNVILKKYRNMVVRACGCH.
```

* * * * *



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ITEM NBR	PATENT NUMBER	FEE CDE	FEE AMT	SUR CHARGE	SERIAL NUMBER	PATENT DATE	FILE DATE	PAY YR	SML ENT	STAT
1	5,258,494	183	1020		07/995,345	11/02/93	12/22/92	04	NO	PAID

ITEM NBR	ATTY DKT NUMBER
1	STK-001CP2DV

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ITEM NBR	PATENT NUMBER	FEE CDE	FEE AMT	SUR CHARGE	SERIAL NUMBER	PATENT DATE	FILE DATE	PAY YR	SML ENT	STAT
1	5,258,494	184	1950	----	07/995,345	11/02/93	12/22/92	08	NO	PAID

ITM NBR	ATTY DKT NUMBER
1	STK-001CP2DV

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COMPARISON OF OP-1 SEQUENCE WITH COP-5 AND COP-7

```
OP-1  LYVSFRDLGWQDWIIAPEGYAAYYCEGECAFPLNSYMNATNHAIVQTLVH
      ||| | | || ||| || || | || ||| |||    | |||| |||||
COP-5 LYVDFSDVGWDDWIVAPPGYQAFYCHGECPFPLADHFNSTNHAVVQTLVN

OP-1  FINPETVPKPCCAPTQLNAISVLYFDDSSNVILKKYRNMVVRACGCH
        |    || || || | ||| || |    | || |  |||  |||
COP-5 SVNSKI-PKACCVPTELSAISMLYLDENEKVVLKNYQEMVVEGCGCR

OP-1  LYVSFRDLGWQDWIIAPEGYAAYYCEGECAFPLNSYMNATNHAIVQTLVH
      ||| | | || ||| || || | || ||| |||    | |||| |||||
COP-7 LYVDFSDVGWNDWIVAPPGYHAFYCHGECPFPLADHLNSTNHAVVQTLVN

OP-1  FINPETVPKPCCAPTQLNAISVLYFDDSSNVILKKYRNMVVRACGCH
        |    || || || | ||| || |    | || |  |||  |||
COP-7 SVNSKI-PKACCVPTELSAISMLYLDENEKVVLKNYQEMVVEGCGCR
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The Effect of Recombinant Human Osteogenic Protein-1 on Healing of Large Segmental Bone Defects*

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Investigation performed at the Department of Orthopaedic Surgery, Tulane University School of Medicine, New Orleans, and Creative Biomolecules, Incorporated, Hopkinton

ABSTRACT: A rabbit ulnar non-union model was used to evaluate the effect of recombinant human osteogenic protein-1 on the healing of a large segmental osteoperiosteal defect. A 1.5-centimeter segmental defect was created in the mid-part of the ulnar shaft of adult rabbits. The defect was filled with an implant containing either recombinant human osteogenic protein-1 or naturally occurring bovine osteogenic protein. The recombinant human osteogenic protein-1 implants consisted of a carrier of 125 milligrams of demineralized, guanidine-extracted, insoluble rabbit bone matrix (the collagen carrier), reconstituted with 3.13, 6.25, 12.5, twenty-five, fifty, 100, 200, 300, or 400 micrograms of recombinant human osteogenic protein-1. Animals that received recombinant human osteogenic protein-1 were compared with animals that received an implant of 250 micrograms of a preparation of naturally occurring bovine osteogenic protein mixed with the collagen carrier. Limbs that served as controls received either the collagen carrier alone or no implant at all. The treated and the untreated defects were examined radiographically and histologically at eight or twelve weeks after implantation. Mechanical testing was performed on six animals.

All implants of recombinant human osteogenic protein-1, except for those containing 3.13 micrograms of the substance, induced complete radiographic osseous union within eight weeks. The defects that were treated with an implant of bovine osteogenic protein also healed within this time-period. The bone induced by both types of implants had new cortices with advanced remodeling and marrow elements. Histological evaluation of this new bone at eight weeks postoperatively revealed primarily lamellar bone, with the formation of new cortices and normal-appearing marrow elements. The average torsional strength and energy-absorption capacity of the union induced by recombinant human osteogenic protein-1 was comparable with that of intact bone. The control defects that had been implanted with collagen carrier alone and those with no implant showed no bridging of the defect.

CLINICAL RELEVANCE: Segmental bone loss and non-union are challenging problems for orthopaedic surgeons. The results of this study demonstrate that a highly purified recombinant human osteogenic protein is capable of inducing healing in a large bone defect in an animal model. The type of implant used in this study may provide an alternative to the use of autogenous graft and allograft bone in the reconstruction of bone defects caused by trauma, neoplasia, or infection. The use of osteogenic proteins to augment or replace bone grafts may reduce the number of operations needed to treat such conditions and may circumvent the risk of transmission of infection that is associated with the transplantation of allografts and autogenous grafts.

Failure of osseous union, whether after a skeletal reconstructive procedure or after a fracture, presents a formidable operative challenge. The use of cancellous or corticocancellous autogenous grafts, or both, has been successful in approximately 80 to 90 per cent of patients[9,11,15,23], but it requires an additional operative procedure to obtain the bone graft, usually from the iliac crest. This additional procedure adds considerable morbidity. Symptoms of pain, hypersensitivity, or anesthesia in the buttocks occur in 6 to 20 per cent of patients, and 3 to 9 per cent have major complications[5,6,38]. In one study, 25 per cent of patients from whom iliac crest grafts had been taken reported substantial pain at an average of five years postoperatively[30].

Allograft bone is often used as an alternative to autogenous bone graft. However, the lower osteogenic potential, the higher rate of resorption, and the inferior revascularization of an allograft compared with an autogenous graft can limit the effectiveness of an allo-

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‡Creative Biomolecules, Incorporated, 35 South Street, Hopkinton, Massachusetts 01748.

graft[6]. The host immunological response may also affect the success of an allograft. Freezing or freeze-drying appears to attenuate this immunological response, but it diminishes the ability of the graft to recruit osteoprogenitor cells and to promote their differentiation[10,16]. Recently, enthusiasm for allograft bone has been tempered by concern about the transmission of infectious agents. Transmission of the human immunodeficiency virus has been reported[19].

In recent years, the search for an acceptable substitute for autogenous bone graft has resulted in an explosion of research involving proteins that induce the formation of bone *in vivo*. The concept of osteogenic bone proteins was described by Urist in 1965[31]. His pioneering work demonstrated the osteogenic potential of demineralized bone segments after implantation at intramuscular sites in animals. Urist postulated that bone matrix must contain a substance or substances that induce the differentiation of pluripotent precursor cells along an osteogenic line.

Numerous bone-inducing proteins have been isolated from bone with the use of a variety of purification procedures. These preparations, known as osteogenic protein, bone morphogenetic protein, osteogenin, and osteoinductive factor, have been found to induce the formation of bone at ectopic sites in animals[3,4,7,20,25,26,28,32]. In addition, preparations of purified bone morphogenetic protein have shown efficacy in the healing of bone defects in studies of animals and humans[8,13,14,17,18,21,29].

With the purification of osteogenic proteins from bone in sufficient quantity and purity to provide data on amino-acid sequences, human cDNAs were isolated and cloned. To date, seven potentially osteogenic proteins have been described[12,36], and three — osteogenic protein-1 (also known as bone morphogenetic protein-7), bone morphogenetic protein-2 (also known as bone morphogenetic protein-2A), and bone morphogenetic protein-4 (also known as bone morphogenetic protein-2B)[12,35] — have been expressed in host cells and have been proved to have osteogenic activity at ectopic sites in animals. The purpose of the present study was to evaluate the use of recombinant human osteogenic protein-1, implanted in combination with an allogeneic-bone collagen carrier, for the restoration of a large segmental defect of the ulnar diaphysis in the rabbit.

Materials and Methods

Preparation of the Implant and Experimental Design

An ulnar segmental-defect model[2] was used to evaluate bone-healing in adult male New Zealand White rabbits that weighed four to five kilograms. The experimental implants of recombinant human osteogenic protein-1 consisted of a carrier of 125 milligrams of demineralized, guanidine-extracted, insoluble rabbit bone matrix (the collagen carrier), reconstituted with 3.13, 6.25, 12.5, twenty-five, fifty, 100, 200, 300, or 400 micrograms of recombinant human osteogenic protein-1. To obtain recombinant human osteogenic protein-1, the full-length human osteogenic protein-1 cDNA sequence encoding 431 amino acids[22] was expressed in cells from Chinese hamster ovaries. The recombinant protein was purified to more than 95 per cent homogeneity from host cell-conditioned media. In an unpublished experiment, the recombinant human osteogenic protein-1 gene-product was implanted subcutaneously in rats to assay its biological activity and was found to induce the formation of ossicles containing lamellar bone and normal-appearing marrow elements. The collagen carrier consisted mainly of type-I rabbit bone collagen, which, when implanted without recombinant human osteogenic protein-1, did not induce the formation of cartilage or bone on subcutaneous implantation in rats. The carrier was prepared by extraction of demineralized rabbit-bone powder with four-molar guanidine hydrochloride, extensive washing of the insoluble matrix with water, and freeze-drying[24].

The animals that received an implant of recombinant human osteogenic protein-1 were compared with the animals that received an implant of 250 micrograms of a preparation of naturally occurring bovine osteogenic protein mixed with the same collagen carrier. The naturally occurring osteogenic protein extracted from bovine cortical bone was partially purified, as previously described[27]. The preparation was obtained from the Sephacryl-300 HR step in the purification protocol and represented a 600-fold purification compared with the crude guanidine-hydrochloride extract. An implant containing recombinant human osteogenic protein-1 was packed into the sites of thirty-five defects, while an implant with bovine osteogenic protein was packed into six sites. Control sites either were implanted with collagen carrier alone (five sites) or were closed without implantation (six sites). Eight forelimbs were left intact (Table I).

The preparations of recombinant human osteogenic protein-1 or bovine osteogenic protein were dissolved, individually, in 1.0 milliliter of 50 per cent acetonitrile containing 0.1 per cent trifluoroacetic acid[27], combined with 125 milligrams of matrix carrier, and freeze-dried.

Operative Procedure

With the rabbit under anesthesia, both forelimbs were shaved and then prepared and draped in a sterile fashion. A lateral incision, approximately 2.5 centimeters in length, was made and the tissues overlying the ulna were dissected. A 1.5-centimeter segmental osteoperiosteal defect was created in the middle of the ulna with an oscillating saw. The radius was left intact for mechanical stability, and no internal or external fixation devices were used. After copious irrigation with saline solution to remove bone debris and spilled marrow cells, the implant of the osteogenic protein and the collagen matrix or the control implant (the collagen matrix) was packed carefully into place to fill the de-

TABLE I
DATA ON THE RABBITS

Animal*	Treatment		Postop. Time to Death (Wks.)
	Right Forelimb*	Left Forelimb*	
Mechanical testing			
1	rh OP-1 (100)	Intact	8
2	rh OP-1 (100)	Intact	8
3	rh OP-1 (100)	Intact	8
4	rh OP-1 (100)	Intact	8
5	rh OP-1 (100)	Intact	8
6	rh OP-1 (100)	Intact	8
Chronic non-union			
7	Matrix alone	Matrix alone	8†
8	rh OP-1 (6.25)	rh OP-1 (6.25)	8†
b OP			
9	Control defect	b OP (250)	8
10	Control defect	b OP (250)	8
11	Control defect	b OP (250)	8
12	Control defect	b OP (250)	12
13	Control defect	b OP (250)	12
14	Control defect	b OP (250)	12
Matrix control			
15	Matrix alone	Intact	12
16	Matrix alone	Intact	12
rh OP-1			
17	Matrix alone	rh OP-1 (6.25)	12
18	rh OP-1 (3.13)	rh OP-1 (3.13)	12
19	rh OP-1 (3.13)	rh OP-1 (3.13)	12
20	rh OP-1 (3.13)	rh OP-1 (3.13)	12
21	rh OP-1 (6.25)	rh OP-1 (12.5)	12
22	rh OP-1 (6.25)	rh OP-1 (6.25)	12
23	rh OP-1 (25)	rh OP-1 (50)	12
24	rh OP-1 (25)	rh OP-1 (50)	12
25	rh OP-1 (25)	rh OP-1 (25)	12
26	rh OP-1 (100)	rh OP-1 (300)	12
27	rh OP-1 (100)	rh OP-1 (300)	12
28	rh OP-1 (200)	rh OP-1 (400)	12
29	rh OP-1 (200)	rh OP-1 (400)	12
30	rh OP-1 (200)	rh OP-1 (400)	12

*rh OP-1 = recombinant human osteogenic protein-1 and b OP = bovine osteogenic protein. The number in parentheses after the type of implant indicates the micrograms of protein implanted at the site.

†The implant was placed eight weeks after the defect had been created, and the animal was killed eight weeks after the implantation.

fect. The soft tissues were closed meticulously in layers to contain the implant. The procedure was repeated on the contralateral side with the appropriate implant. Postoperatively, all animals received 200,000 units of procaine penicillin G and 250 milligrams of dihydrostreptomycin per day by intramuscular injection. The animals were allowed full weight-bearing activity, water, and rabbit chow (Ralston Purina, Richmond, Indiana) *ad libitum*.

An additional two animals (Animals 7 and 8), which had received no implant at the time of the initial procedure, had implants placed eight weeks later to simulate a chronic non-union. At the time of the operation, all fibrous tissue was resected from the site of the non-union and the ends of the bone were freshened before placement of the implant. One rabbit (two sites) received implants containing 6.25 micrograms of recombinant human osteogenic protein-1, and the other rabbit (two sites) received control implants of collagen carrier.

Evaluation of the Results

Radiographs of the forelimbs were made weekly until the time of death at eight or twelve weeks postoperatively, at which time the ulna and radius from both forelimbs were excised *en bloc* and photographed. Radiographs were also made after all of the soft tissues had been stripped.

Non-decalcified ground histological and corresponding microradiographic sections and non-decalcified microtome sections were prepared from the site of the defect and from adjacent normal bone. Ground histological sections were stained with basic fuchsin and toluidine blue, and non-decalcified microtome sections were stained with von Kossa stain, Goldner trichrome stain, or toluidine blue.

Mechanical testing was performed on both ulnae of six animals (Animals 1 through 6) eight weeks after implantation. In these animals, the operation had been performed only on the right forelimb; the contralateral side had been left intact to serve as a control limb. The experimental implants for these animals consisted of 125 milligrams of collagen carrier reconstituted with 100 micrograms of recombinant human osteogenic protein-1. Immediately after death, the forelimbs were harvested and stripped of all overlying tissues. The ulna was isolated carefully from the radius, and the specimens were maintained in normal saline solution until testing, which was completed immediately after death. Torsional testing to failure was performed by placement of each end of the bone into a cylindrical aluminum sleeve; then, with one end fixed, the other end was rotated. Since the ulna of a rabbit is slightly curved, the specimens were mounted eccentrically to keep the axis of ulnar rotation coaxial with that of the testing device. The torsional force was applied at a rate of fifty millimeters per minute, with a lever arm of six centimeters, by a servohydraulic materials-testing system (MTS, Minneapolis, Minnesota). Force-angular displacement curves were generated, from which the torque and angular deformation to failure were obtained, and the energy absorbed to failure was calculated as the area under the curve.

Results

Radiographic Results

All of the implants of recombinant human osteogenic protein-1 except for those containing 3.13 micrograms of the protein induced complete radiographic osseous union across the defect within eight weeks. All defects were filled with new bone of uniform radiodensity, which approximated the radiodensity of normal bone. The defects that had been filled with implants of

bovine osteogenic protein also healed within this period. In the defects that had been filled with collagen carrier alone and in the untreated control defects, two to three millimeters of callus occasionally formed at the free bone ends, but it failed to bridge the defect at postoperative intervals of as many as twelve weeks (Fig. 1). There were no radiographic differences between the sites that had been filled with collagen carrier alone and those that had not received an implant.

A distinct radiographic progression of defect-healing was observed with the implants of recombinant human osteogenic protein-1 and those of the bovine osteogenic protein (Figs. 2-A through 3-D). Immediately postoperatively, the entire area of the defect was radiolucent. After two weeks, the site of the defect had a speckling of radiopaque material and, by three to four weeks, the entire defect was filled with dense material. Remodeling was evident at four to five weeks, with the formation of new cortices and a medullary canal. Bone-remodeling continued until the rabbits were killed, with the new cortices reaching normal radiographic size and density at about eight weeks. Additional remodeling was seen in the animals that were examined at twelve weeks. Greater amounts of implanted recombinant human osteogenic protein-1 resulted in correspondingly more formation of bone and a faster rate of healing, but the final radiographic result at eight weeks was comparable for all animals that had an implant of recombinant human osteogenic protein-1 (except for those containing 3.13 micrograms of protein). The threshold value for complete osseous union with remodeling was 6.25 micrograms of recombinant human osteogenic protein-1, with the quantity and rapidity of the formation of bone similar to that seen with the bovine osteogenic protein implants.

FIG. 1

Radiograph of the forelimb of an untreated control animal, made at twelve weeks, showing a persistent non-union and minimum new-bone formation.

Gross and Histological Results

The gross appearance of the defects that had been treated with recombinant human osteogenic protein-1 paralleled the radiographic appearance (Fig. 4): new cortices with a width, contour, and density that were similar to the normal cortices were seen. The over-all dimensions of the mass of new bone occasionally exceeded that of the normal ulna. The sites that had been treated with bovine osteogenic protein appeared very similar to those that had been treated with recombinant human osteogenic protein-1. Fibrous tissue was observed in all control defects. The histological appearance of all of the sites that had been implanted with recombinant human osteogenic protein-1 (Fig. 5-A), except for those implanted with 3.13 micrograms, was similar to that of the sites that had been implanted with bovine osteogenic protein (Fig. 5-B).

The animals that were killed at eight weeks had new cortices composed primarily of lamellar bone but with some areas of irregular woven bone on histological examination. Increased amounts of recombinant human osteogenic protein-1 resulted in the earlier formation of a substantial mass of new bone. However, by twelve weeks, all sites had reached a similarly high degree of remodeling. The medullary canal was well formed and contained normal-appearing marrow elements (Fig. 6). Neither areas of new cartilage formation nor remnants of the collagen carrier were observed. The sites of the control defects had some small areas of woven bone at the cut ends of the cortices but there was only fibrous tissue within the defect (Fig. 5-C). Remnants of the collagen carrier were observed in some of the control specimens that had been treated with the collagen carrier.

Chronic Sites of Non-Union

The two animals in which a chronic non-union had been created had radiographic, gross, and histological evidence of healing that was indistinguishable from that of the animals in which recombinant human osteogenic protein-1 had been implanted at the initial operative procedure. The control sites demonstrated the same proliferation of fibrous tissue and lack of healing seen in other control defects.

FIG. 2-A

Figs. 2-A through 2-D: Anteroposterior radiographs of an ulna that was treated with 12.5 micrograms of recombinant human osteogenic protein-1.

Fig. 2-A: Immediately after the implantation.

FIG. 2-B

Two weeks after the implantation, there is abundant new-bone formation and early cortical bridging.

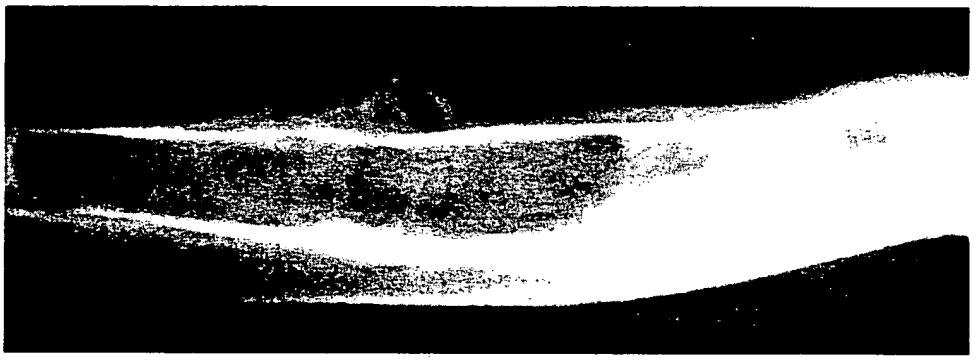

FIG. 2-C

Six weeks after the implantation, new cortices are evident.

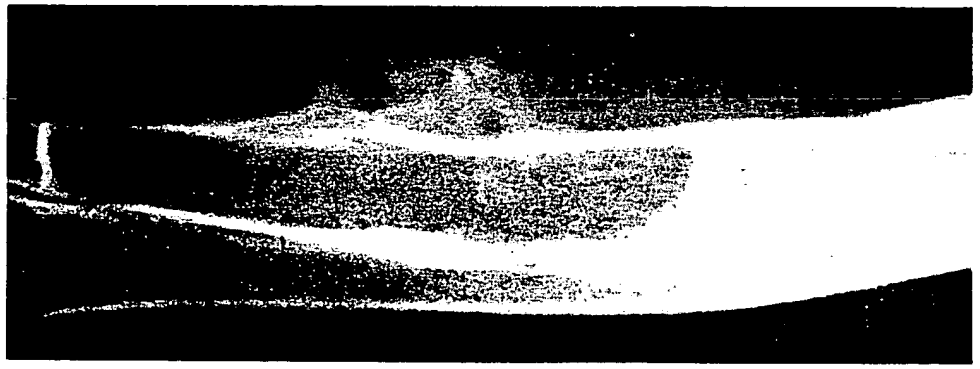

FIG. 2-D

Eight weeks after the implantation.

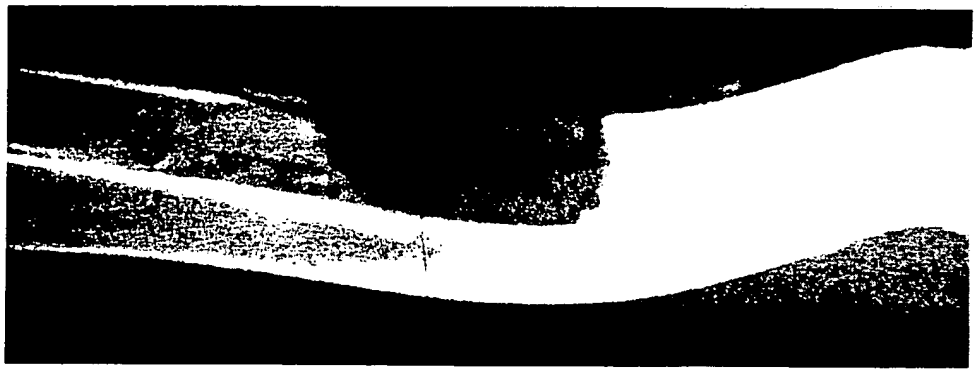

FIG. 3-A

Figs. 3-A through 3-D: Anteroposterior radiographs of an ulna that was treated with bovine-derived osteogenic protein. The radiographic progression was similar to that seen for the defects that were treated with recombinant human osteogenic protein-1.

Fig. 3-A: Immediately after the implantation.

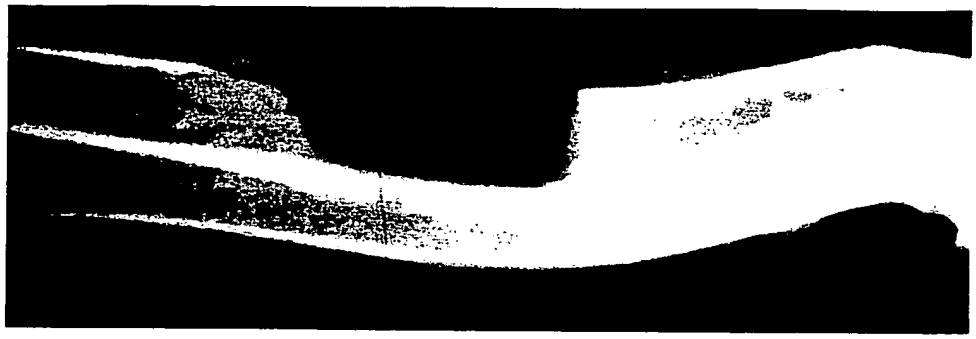

FIG. 3-B

Two weeks after the implantation.

FIG. 3-C

Six weeks after the implantation.

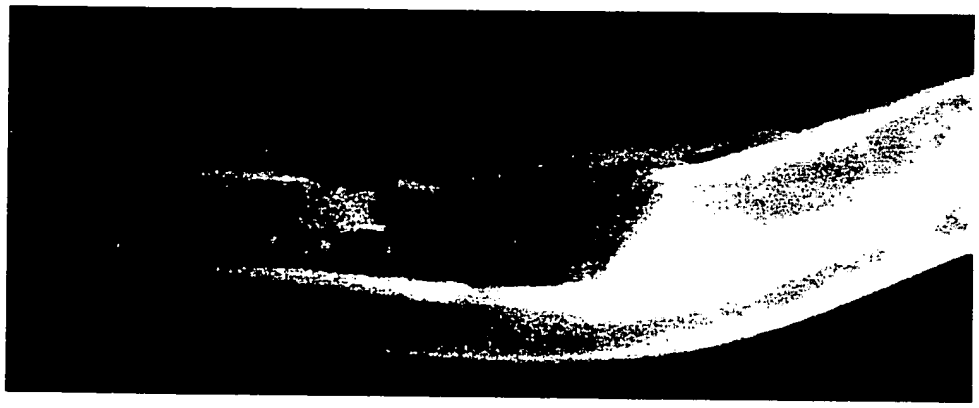

FIG. 3-D

Eight weeks after the implantation.

TABLE II
MECHANICAL TEST DATA

	Maximum Torque*			Angular Deformity*			Energy Absorption*		
	Control (Nm)	Implant (Nm)	Implant Compared with Control (Per cent)	Control (Degrees)	Implant (Degrees)	Implant Compared with Control (Per cent)	Control (Nm/Degrees)	Implant (Nm/Degrees)	Implant Compared with Control (Per cent)
Animal									
1	0.35	0.35		20.9	12.6		4.07	2.88	
2	0.40	0.62		14.3	19.3		4.51	7.25	
3	0.64	0.30		24.6	13.4		8.64	2.94	
4	0.51	0.38		15.1	13.4		4.66	3.34	
5	0.38	0.54		16.7	21.8		4.18	7.09	
6	0.35	0.30		16.8	19.3		4.42	5.25	
Average and stand. dev.	0.44 ± 0.12	0.42 ± 0.13		18.1 ± 3.9	16.6 ± 4.0		5.08 ± 1.76	4.79 ± 2.26	
			95 (p = 0.79†)			92 (p = 0.64†)			94 (p = 0.84†)

*To failure of the specimen.
†Based on paired Student t test. The p values reflect a lack of significant difference between the intact left ulna (control) and the right ulna, which contained a defect in which 100 micrograms of recombinant human osteogenic protein-1 in 125 milligrams of collagen carrier had been implanted.

Mechanical Testing

The average torque to failure of the ulnae in which recombinant human osteogenic protein-1 had been implanted was 95 per cent (range, 47 to 155 per cent) that of the intact side. The average angular deformation to failure was 92 per cent (range, 54 to 135 per cent) that of the intact side. The average energy absorption to failure was 94 per cent (range, 34 to 170 per cent) that of the intact side (Table II). All specimens failed near the middle of the bone in a spiral fracture pattern that was consistent with the torsional load applied. The specimens implanted with recombinant human osteogenic protein-1 failed in a pattern similar to that of the normal, untreated control specimens.

The torque-to-failure values obtained with our method for both the experimental and the control specimens were generally lower than those seen in earlier studies on rabbit bone[2]. This is probably attributable to the relatively slow speed at which we applied the torsional load compared with the impulse load used in other studies.

Discussion

The most recent advance in the development of osteogenic proteins is the cloning and expression of recombinant forms of human proteins. The human osteogenic protein-1 gene has been expressed in cultures of cells from monkey kidneys and Chinese hamster ovaries, and the recombinant human osteogenic protein-1 gene product has been characterized[22]. After purification to more than 95 per cent homogeneity, the recombinant human osteogenic protein-1 was tested *in vivo* in a rat ectopic bone-formation assay in preparation for the present study. When combined with rat bone collagen matrix as the carrier, only nanogram quantities of recombinant human osteogenic protein-1 induced formation of fully matured marrow-containing ossicles. The half-maximum bone-inducing activity of recombinant human osteogenic protein-1 was equivalent to that of highly purified bovine osteogenic protein. The sequence of cellular events involved in the endochondral formation of bone induced by recombinant human osteogenic protein appears to be identical to that induced by de-

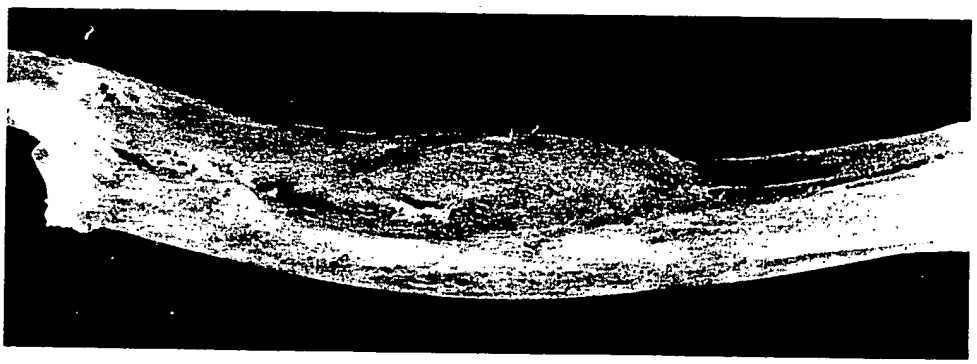

FIG. 4

Photograph of the bones of the forelimb, revealing osseous union of the ulna (top) eight weeks after implantation of 12.5 micrograms of recombinant human osteogenic protein-1.

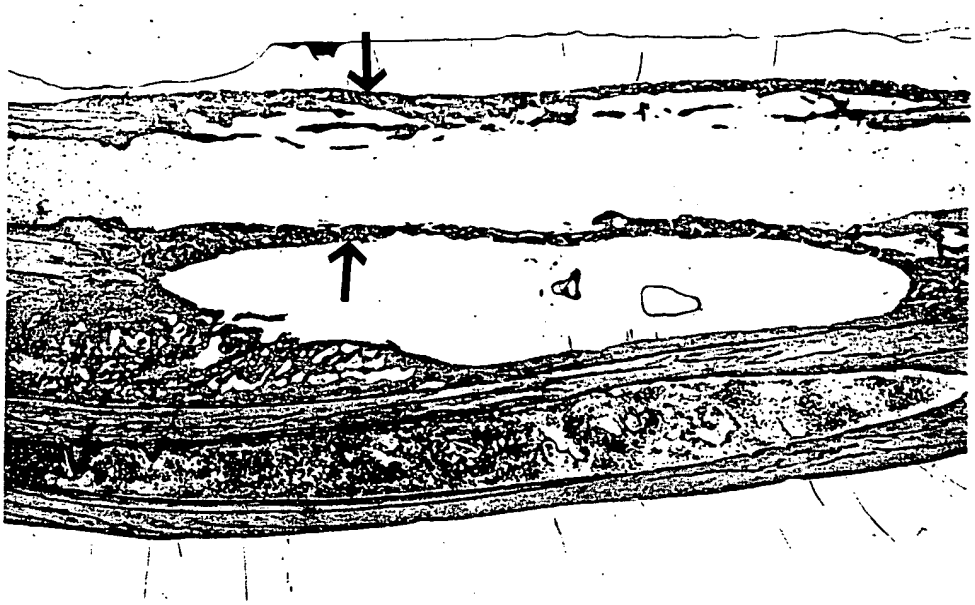

FIG. 5-A

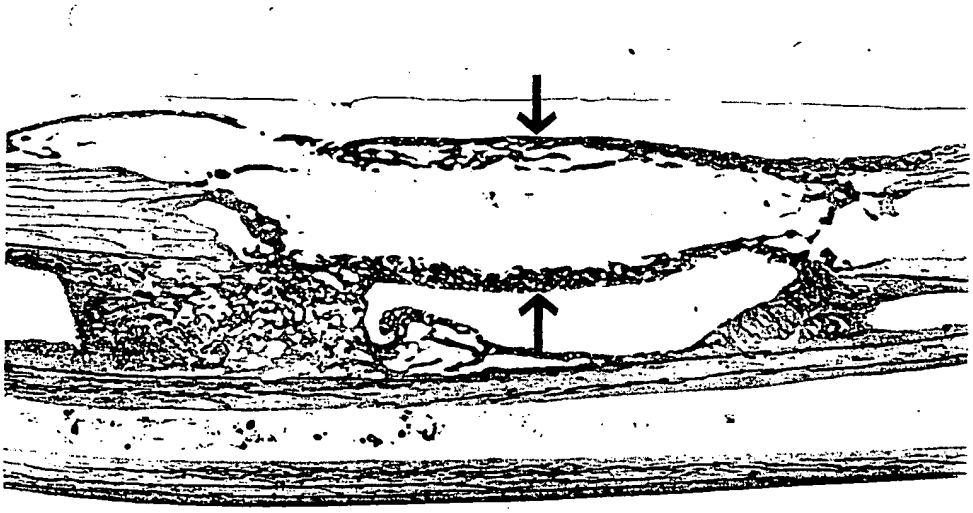

FIG. 5-B

Figs. 5-A, 5-B, and 5-C: Low-power photomicrographs of a specimen treated with 12.5 micrograms of recombinant human osteogenic protein-1 (Fig. 5-A), one treated with bovine osteogenic protein (Fig. 5-B), and a control specimen (no implant)(Fig. 5-C), all made at twelve weeks after the implantation (toluidine blue and basic fuchsin, × 2).

Figs. 5-A and 5-B: There is formation of new cortices and a medullary canal (arrows) in the defects that were treated with recombinant human osteogenic protein-1 or bovine osteogenic protein.

mineralized bone matrix or by naturally occurring osteogenic protein.

The present study demonstrates that a highly purified recombinant human osteogenic protein is capable of inducing healing in a bone defect in an animal model. The biological effect of recombinant human osteogenic protein-1 is dependent on the amount that is implanted, and the effect is at least equal to that exhibited by a preparation of naturally occurring bovine osteogenic protein. At eight weeks postoperatively, histological evaluation of the new bone formed at the sites of the defects in which recombinant human osteogenic protein-1 had been implanted revealed primarily lamellar bone, with formation of new cortices and normal-appearing marrow elements. The average torsional strength and energy-absorption capacity of the united bone were comparable with those of the intact bone.

Implants of Demineralized Bone Matrix

A substantial amount of basic and clinical research

FIG. 5-C

There is a lack of bridging in the control defect.

has provided the foundation for recent achievements in the field of osteogenic implants. A brief review of this previous work is helpful to place the present work into an appropriate historical perspective.

As early as the late nineteenth century, it was recognized that implants of demineralized bone matrix were effective in the reconstruction of bone defects, which were frequently associated with infection[33]. While osteoinductive implants of demineralized bone have recently been used primarily in craniomaxillofacial reconstruction[6], a number of studies have demonstrated the potential of such implants in the treatment of segmental defects of long bones.

Einhorn et al. demonstrated that, after twelve weeks, implants of demineralized bone matrix led to the healing of large diaphyseal defects in a rat femoral non-union

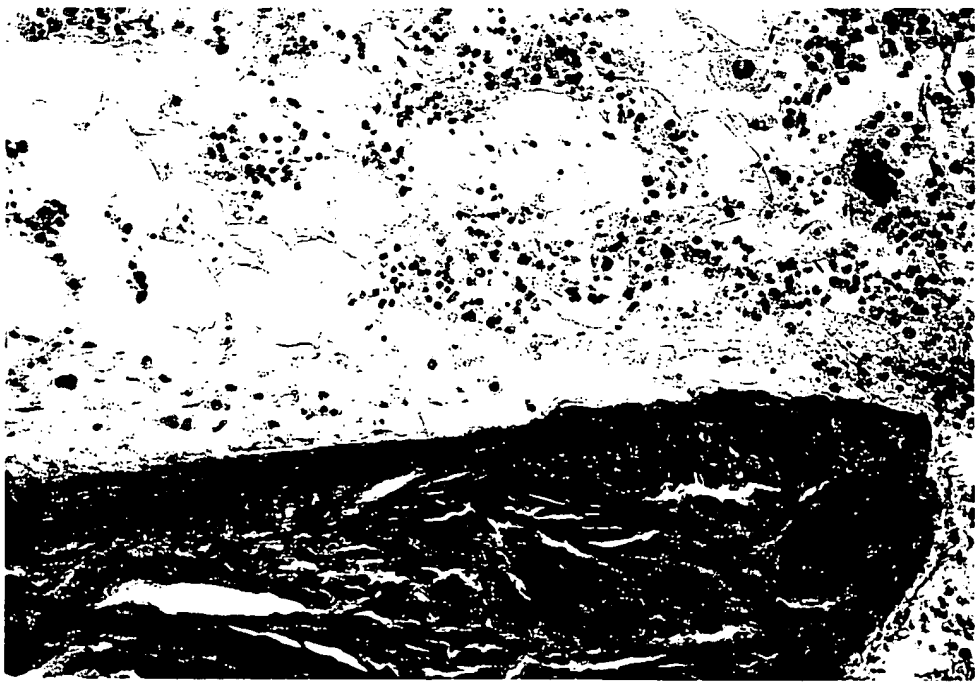

FIG. 6

High-power photomicrograph of a defect that was treated with recombinant human osteogenic protein-1, made eight weeks after implantation, revealing a mixture of woven and lamellar bone with normal-appearing marrow cells (Goldner trichrome, × 250).

model[8]. The stiffness and energy-absorbing capacity of the healed femora were comparable with those of intact bone, and the torsional strength was 35 per cent of intact bone. Bolander and Balian showed, with analysis of a model similar to the one used in the current study, that implants of autogenous demineralized bone matrix induced healing that was equivalent to that obtained with untreated corticocancellous autogenous grafts, but mechanical testing with both experimental groups showed approximately 50 per cent of the strength of intact bone at twelve weeks[2]. While the sites of the defects in that study were filled with new bone, it was mainly woven bone with little remodeling. Our radiographic and histological data indicate that the healing induced by implants of recombinant human osteogenic protein-1 may be superior to that induced by demineralized bone matrix. By eight weeks, the sites of the defects that had contained implants of recombinant human osteogenic protein-1 were filled with primarily lamellar bone with a well developed medullary canal. The high average strength of union at this time was consistent with the presence of extensive remodeling. The experimental sites were 95 per cent as strong as the untreated control sites at eight weeks and stronger than the previously reported results obtained at twelve weeks with implants of demineralized bone matrix and autogenous grafts[2].

Characterization of Partially Purified Osteogenic Proteins

As far as we know, the solubilization and extraction of bone morphogenetic proteins was first reported in 1979 by Urist et al.[33]. The technique described in their report used four-molar guanidine hydrochloride to solubilize the non-collagenous matrix proteins, followed by dialysis against water. The product showed increased osteogenic activity compared with that of demineralized bone matrix alone. In 1981, a report by Sampath and Reddi demonstrated that the bone-matrix residue that was present after the extraction was non-osteogenic in a rat *in vivo* ectopic bone-formation assay but its osteogenicity could be totally restored by reconstitution of the matrix with the extract[24]. The assay, in which test samples were implanted subcutaneously in allogeneic rats, has become the standard method for assessment of the osteogenic activity of protein preparations[1].

Partially purified bone morphogenetic proteins have been tested extensively in animal bone-healing models, as well as in the ectopic bone-formation assay. Nilsson et al. demonstrated the success of bovine bone morphogenetic protein in a canine ulnar diaphyseal non-union model[21]. Their study demonstrated that the preparation of bone morphogenetic protein, which was implanted with gelatin, induced healing of the bone defect during a twelve-week period, independent of bone matrix. Heckman et al. used a canine radial diaphyseal defect model in which they treated twelve-week-old non-unions with partially purified canine bone morphogenetic protein on a polylactic-acid carrier[14]. This method more closely simulates the clinical problem of non-union, whereas earlier studies simulated primary treatment of bone loss. The preparation of bone morphogenetic protein induced bridging of the defects by trabecular bone at twelve weeks[14].

In the present study, the defects that were packed with naturally occurring bovine osteogenic protein also healed. However, 6.25 micrograms of recombinant human osteogenic protein-1 was found to have activity that was equivalent to 250 micrograms of bovine osteogenic protein. Important advantages to the use of highly purified recombinant proteins include the elimination of the risk of transmission of infection, which is associated with the transplantation of natural bone extracts, and the avoidance of possible immunogenic problems arising from the implantation of impure products.

Partially Purified Human Bone Morphogenetic Proteins

Preliminary studies have been done with use of partially purified, naturally occurring human bone morphogenetic proteins in the clinical treatment of non-unions[17,18]. Six patients who had an established tibial non-union after failure of internal or external fixation were managed with autogenous bone-grafting and internal or external fixation, augmented with implants of human bone morphogenetic protein on a polylactic acid-polyglycolic acid carrier. Union was achieved in all patients at an average of 5.7 months[18]. Twelve patients who had a femoral non-union that was refractory to standard measures were managed with various combinations of internal fixation and autogenous bone grafts or allografts in addition to implants of human bone morphogenetic protein on a carrier of either gelatin or polylactic acid-polyglycolic acid. Eleven of the twelve patients had union at an average of 4.7 months[17]. No major complications or adverse reactions were observed. While no definitive conclusions can be drawn, since bone grafts were used and the studies were uncontrolled, these studies demonstrated the safety of the implants in humans. The investigators were confident that the human bone morphogenetic protein had played an important role in the healing of these long-standing non-unions.

Recombinant Human Osteogenic Proteins

In addition to the recombinant human osteogenic protein-1 that we studied, other recombinant human osteogenic proteins have been isolated and tested. Wang et al. recently reported the expression of the human bone morphogenetic protein-2 (also known as bone morphogenetic protein-2A) gene in cells from Chinese hamster ovaries[35]. Their recombinant bone morphogenetic protein-2A homodimer was also active in the rat ectopic bone-formation assay. However, the data showed that approximately tenfold more of the 50 per cent pure recombinant bone morphogenetic protein-2A was necessary to achieve the same level of osteo-

genic activity observed with the corresponding highly purified bovine preparation[35]. Recently, Yasko et al. reported the healing of femoral non-unions with recombinant human bone morphogenetic protein-2 in rats[37]. Hammonds et al. reported the expression of the bone morphogenetic protein-2B (also known as bone morphogenetic protein-4) gene in cultures of human kidney cells, and they found that the gene product induced new-bone formation at an ectopic site[12].

In summary, our study demonstrates that the implantation of recombinant human osteogenic protein-1 leads to the healing of bone defects in an animal model by the induction of new bone with an appearance and mechanical performance equivalent to that induced by naturally occurring bone extracts. The use of highly purified recombinant forms of osteogenic proteins will eliminate the risk of transmission of infection and will circumvent possible immunogenic problems that are associated with the implantation of impure materials. The recombinant human osteogenic protein-1 implant may provide an alternative to the use of autogenous graft and allograft bone in the reconstruction of bone defects caused by trauma, neoplasia, or infection. Osteogenic protein-1 may also prove to be useful in prosthetic fixation, spinal arthrodesis, and the primary treatment of fractures.

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Use of recombinant human osteogenic protein-1 for the repair of subchondral defects in articular cartilage in goats

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Abstract: The objective of this pilot study was to examine *in vivo* the potential of recombinant human osteogenic protein-1 (rhOP-1, also called bone morphogenetic protein-7, BMP-7) for treatment of subchondral lesions by induction of new hyaline cartilage formation. Subchondral left knee defects in 17 mature goats were treated with fresh coagulated blood mixed with (1) rhOP-1 combined with collagen (OP-1 device, 400 μg/mL); (2) rhOP-1 alone (OP-1 peptide, 200 μg/mL); (3) OP-1 device with small particles of autologous ear perichondrium; (4) OP-1 peptide with small particles of autologous ear perichondrium; or (5) autologous ear perichondrium alone (controls). rhOP-1 was combined with either collagen (OP-1 device) or not (OP-1 peptide). The defects were closed with a periosteal flap. The formation of cartilage tissue was studied by histologic and biochemical evaluation at 1, 2, and 4 months after implantation. One and 2 months after implantation there were no obvious differences between control and rhOP-1-treated defects. Four months after implantation, only one out of three controls (without rhOP-1) showed beginning signs of cartilage formation while all four rhOP-1-treated defects were completely or partly filled with cartilage. A significant linear relationship was found between rhOP-1 concentration and the total amount of aggrecan in the defects. These results suggest that implantation of rhOP-1 promotes cartilage formation in subchondral defects in goats at 4 months after implantation. Therefore, rhOP-1 could be a novel factor for regeneration of cartilage in articular cartilage defects.
 J Biomed Mater Res, 49, 506–516, 2000.

Key words: cartilage; growth factors; tissue engineering; osteogenic protein-1; cartilage repair

INTRODUCTION

Articular cartilage defects are a common problem in orthopedic practice. The spontaneously regenerated cartilage after traumatic or osteoarthritic defects of hyaline cartilage has proven to be of limited quality.[1–3] Injuries of articular cartilage do not heal and usually progress to degeneration. A limiting factor in the response of articular cartilage to injury is the low mitotic activity in mature articular cartilage, which is in contrast to the rapid chondrocyte mitosis during normal cartilage growth. The mature chondrocytes within the tissue also have limited capacity for increasing matrix synthesis; they do not synthesize sufficient matrix to repair significant large-tissue defects. Therefore the introduction of cells with a strong chrondrogenic potential could be an option in the repair of articular cartilage defects.

A source of cells with chondrogenic potential is the perichondrium tissue. The use of perichondrium, which has the ability to produce new cartilage, as has been verified in several experimental and clinical studies,[4–6] seems promising in the repair of hyaline cartilage defects. The cartilaginous potential remains when the perichondrial stem cells are grafted to other parts of the body. Clinically, perichondrial grafting has been proven successful in many cases.[4,7–9] The

No benefit of any kind will be received either directly or indirectly by the authors.

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advantage of biologic resurfacing using perichondrium is the possibility of obtaining hyaline cartilage.[10]

To enhance the chondrogenic potential of the perichondrium, biotechnology now provides access to new cartilage repair concepts via administration of bone morphogenetic proteins (BMPs). The discovery of a family of BMPs that can induce enchondral bone formation in adult animals suggests that BMP-derived inductive signals may be intimately involved in both cartilage and bone formation. Recombinant human osteogenic protein-1 (rhOP-1, also called BMP-7) has been shown capable of restoring large diaphyseal defects in non human primates,[11] demonstrating its therapeutic potential as an anabolic agent in bone repair. In addition, *in vitro* observations suggest that rhOP-1 may be effective in stimulating the formation of cartilage at sites of articular injury. In cultured primitive anlagen of embryonic long bones, chondrogenesis in the bone ends and the future joint ends was strongly enhanced by rhOP-1 treatment,[12] suggesting that rhOP-1 stimulates the differentiation of chondroprogenitor cells into cartilage. We recently have demonstrated that one single dosage of rhOP-1 stimulates chondroblastic differentiation in an explant consisting of goat perichondrium tissue embedded in autologous blood and cultured for 3 weeks.[13] This study suggested that rhOP-1 has a direct stimulating effect on the formation of cartilage by stem cells and therefore may be used for the production of cartilage *in vivo*. Based on these results, the present study was designed to examine the influence of rhOP-1 on cartilage formation in the repair of subchondral articular cartilage lesions in mature goats. The hypothesis was tested that implantation of rhOP-1 in the lesion would enhance the formation of hyaline cartilage, as evaluated by histologic and biochemical methods.

MATERIALS AND METHODS

Implant materials

rhOP-1 kindly was provided by Stryker Biotech (Natick, Massachusetts; manufactured by Creative Biomolecules, Hopkinton, Massachusetts). Implants consisted of fresh coagulated autologous blood mixed with (1) rhOP-1 combined with collagen (OP-1 device); (2) rhOP-1 alone (without collagen; OP-1 peptide); (3) OP-1 device with small particles of autologous ear perichondrium; (4) OP-1 peptide with small particles of autologous ear perichondrium; or (5) autologous ear perichondrium alone (controls). The concentration of rhOP-1 was 400 μg/mL in the device group and 200 μg/mL in the peptide group. The goats were sacrificed and the operated knees were analyzed at 1, 2, and 4 months after implantion (Table I). These time points were chosen based on data from other studies with rabbits.[5,6]

TABLE I.
Design of the Study

Treatment of Goats	Implantation Time (Months)		
	1	2	4
RhOP-1 device +perichondrium	1	1	1
RhOP-1 device −perichondrium	1	1	1
RhOP-1 peptide +perichondrium	1	1	1
RhOP-1 peptide −perichondrium	1	1	1
Control +perichondrium	1	1	3

Numbers indicate the size of each group: rhOP-1 device-rhOP-1 with collagen; rhOP-1 peptide-rhOP-1 without collagen; +perichondrium-with morselized perichondrium; −perichondrium-without perichondrium.

Preparation of implants

The procedure of the preparation of the implants is summarized in Figure 1. Autologous perichondrium tissue was obtained from outer ear perichondrium of a goat. The perichondrial tissue was dissected and weighed. Then the tissue was morselized in particles of less than 1 mm in diameter. The morselized tissue was transferred to one well of a 96-well culture dish (Greiner, Alphen a/d Rijn, The Netherlands) at 40 mg. Subsequently, 5 to 10 mL of fresh autologous blood were taken from the same goat during surgery and immediately added to the well using a syringe. The required amount of rhOP-1 (device or peptide) was added,

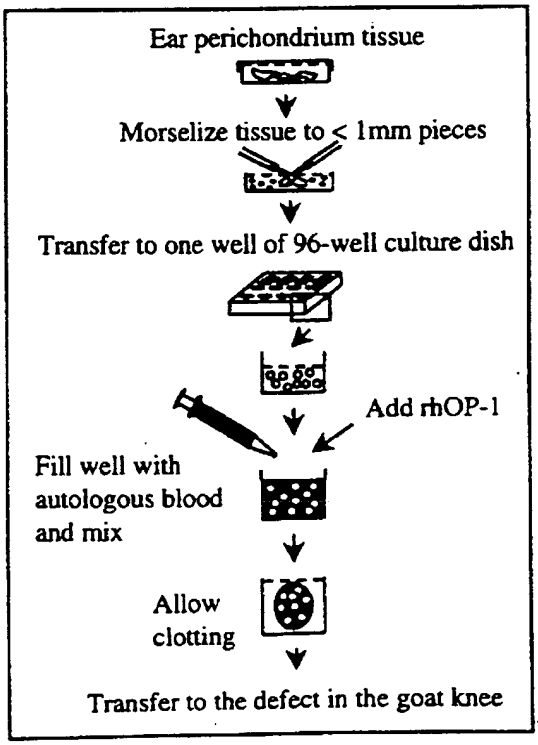

Figure 1. Flow diagram showing the preparation of implants. rhOP-1 was added once, when preparing the implant.

TABLE II
Modified Histologic Scoring System (Moran et al 1992 (14), O'Driscoll et al. 1986 (15), Takahashi et al. 1995 (16), Noguchi et al. 1994 (17), Wakitani et al. 1994 (18)).

Category	Points
Surface irregularity	
Smooth >¾	3
Moderate >½–¾	2
Irregular, ¼–½	1
Severely irregular <¼	0
Bonding to adjacent cartilage	
Bonded at both ends of the graft	2
Bonded at one end or partially at both ends	1
Not bonded	0
Regenerated subchondral bone	
Complete	2
Partially	1
None	0
Inflammatory cell infiltration around implant	
None	2
Partially	1
Complete	0
Chondrocyte clustering	
None	2
<25% of cells	1
25% to 100% of cells	0
Freedom from degenerative changes in adjacent cartilage	
Normal cellularity, no clusters, normal staining	3
Normal cellularity, mild clusters, moderate staining	2
Mild or moderate hypocellularity, slight staining	1
Severe hypocellularity, poor or no staining	0
Metachromasia	
Normal or near normal	3
Moderate	2
Slight	1
None	0
Hyalinity	
Hyaline cartilage	4
Mostly hyaline cartilage	3
Mostly fibrocartilage	2
Mostly noncartilage	1
Noncartilage only	0
Maximum score	21

Histologic analysis was performed on undecalcified sections stained with toluidine blue or Goldner's trichrome method.

and the content of the well was mixed thoroughly with a spatula and allowed to clot. The blood clot, plus additives, was used for autologous implantation within 30 min after preparation of the implant. The total volume of the implant after clotting was 150 μL.

Test animals

Seventeen skeletal mature female Dutch milk goats, approximately 2 years old and weighing approximately 50 kg, were used. The maturity of the skeleton was confirmed in several identical fully grown female goats by radiographic analysis of the growth areas. Assignment of the goats to groups was done at random. The goats were held in pens under identical conditions with a normal diet. Unrestricted weight-bearing and activity was allowed as tolerated post operatively. The experimental protocol was approved by the Committee for Animal Welfare of the Vrije Universiteit.

Surgery

When perichondrium was used for implantation, the perichondrium tissue of the left ear was first exposed via a longitudinal dorsal incision under general anesthesia and routine aseptic conditions. A flap of perichondrium tissue approximately 2 × 3 cm^2 was removed from the dorsal side of the ear, and the ear wound was closed. Under sterile conditions the perichondrium tissue was used for preparation of the implant. Then, via a medial parapatellar approach, the left knee was opened. The patella was dislocated to the lateral side and the medial condyle was exposed. With a sharp hollow tube the outlines of a standardized 0.9-cm diameter were made in the anterior weight-bearing part of the medial condyle. Using a hand burr that was placed inside the tube, a defect through the subchondral bone was created. Care was taken to create sharp edges in the cartilage. The defect was deepened until the subchondral bone was reached. Then the distal medial femur was exposed and a periosteal flap, which was slightly larger in diameter than the defect in the medial condyle, was taken. The periosteal flap, with its cambium layer towards the defect, was partly sutured to the margins of the defect with non absorbable polypropylen (Prolene) 5/0. After lavage of blood and cartilage remnants, the defect was filled with the prepared implant and the suturing of the periosteal flap was completed. The sutures were checked by several flexions of the knee. The patella then was reduced and the knee was closed in layers.

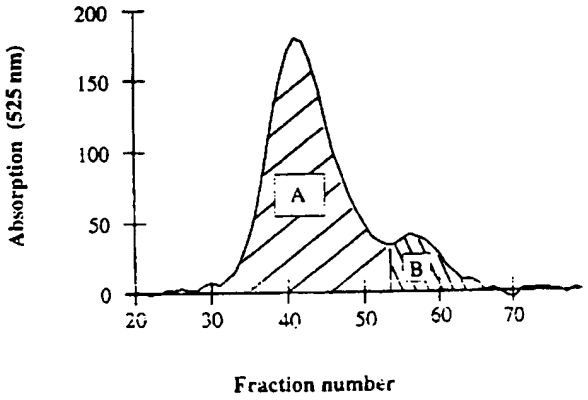

Figure 2. Example of an elution profile on sepharose CL-2B of healthy articular cartilage. Peak A represents large proteoglycans (aggrecan); peak B represents small proteoglycans. The area under peak A, divided by the total area of peaks A plus B, represents the percentage aggrecan of the sample.

TABLE III
Microscopic Appearance of All Defects, Based on the Histologic Scoring System of Table II. A Perfect Score For All Items Is 21 Points.

Category*	Implantation Time (Months)	Surface Irregularity	Bonding to Adjacent Cartilage	Regenerated Subchondral Bone	Inflammatory Cell Infiltration Around Implant	Chondrocyte Clustering	Freedom From Degenerative Changes in Adjacent Cartilage	Metachromasia	Hyalinity	Total
Control	1	0	0	0	2	0	1	0	0	4
Control	2	3	1	1	2	1	3	1	2	14
Control	4	0	0	1	0	0	1	0	0	2
Control	4	2	2	2	1	0	2	0	0	9
Control	4	1	1	1	2	1	3	2	2	13
OP-1 pep	1	3	1	1	2	0	2	0	0	9
OP-1 pep	2	3	1	1	2	0	2	2	3	14
OP-1 pep	4	3	1	1	2	1	3	2	2	15
rhOP-1 pep + perich	1	3	2	1	2	0	2	1	1	12
rhOP-1 pep + perich	2	3	1	1	2	1	3	1	2	14
rhOP-1 pep + perich	4	1	1	1	2	1	2	1	2	11
rhOP-1 dev	1†	-	-	-	-	-	-	-	-	-
rhOP-1 dev	2	2	2	1	2	1	2	1	1	12
rhOP-1 dev	4	0	1	1	2	0	2	2	2	10
rhOP-1 dev + perich	1	1	0	1	2	1	3	1	2	11
rhOP-1 dev + perich	2	1	1	1	2	1	2	2	2	12
rhOP-1 dev + perich	4	3	1	1	2	0	2	3	3	15
Maximum score		3	2	2	2	2	3	3	4	21

rhOP-1 dev-rhOP-1 device; + perich-with perichondrium; rhOP-1 pep-rhOP-1 peptide. *Categories of the histologic evaluation: †infected.

Sample harvesting

One, 2, and 4 months after implantation, the animals were sacrificed and the operated joint reopened. Gross morphology of the joint was scored, using the method of Moran et al.[14] The medial condyle containing the cartilage defect was removed and divided into two similar halves using a cooled diamond string saw (Rowa Techniek B.V., Leiderdorp, The Netherlands), one for histology and one for biochemistry. Undamaged control articular cartilage also was taken from the medial condyle of the same knee joint for biochemical analysis. During this procedure the tissue was kept moist

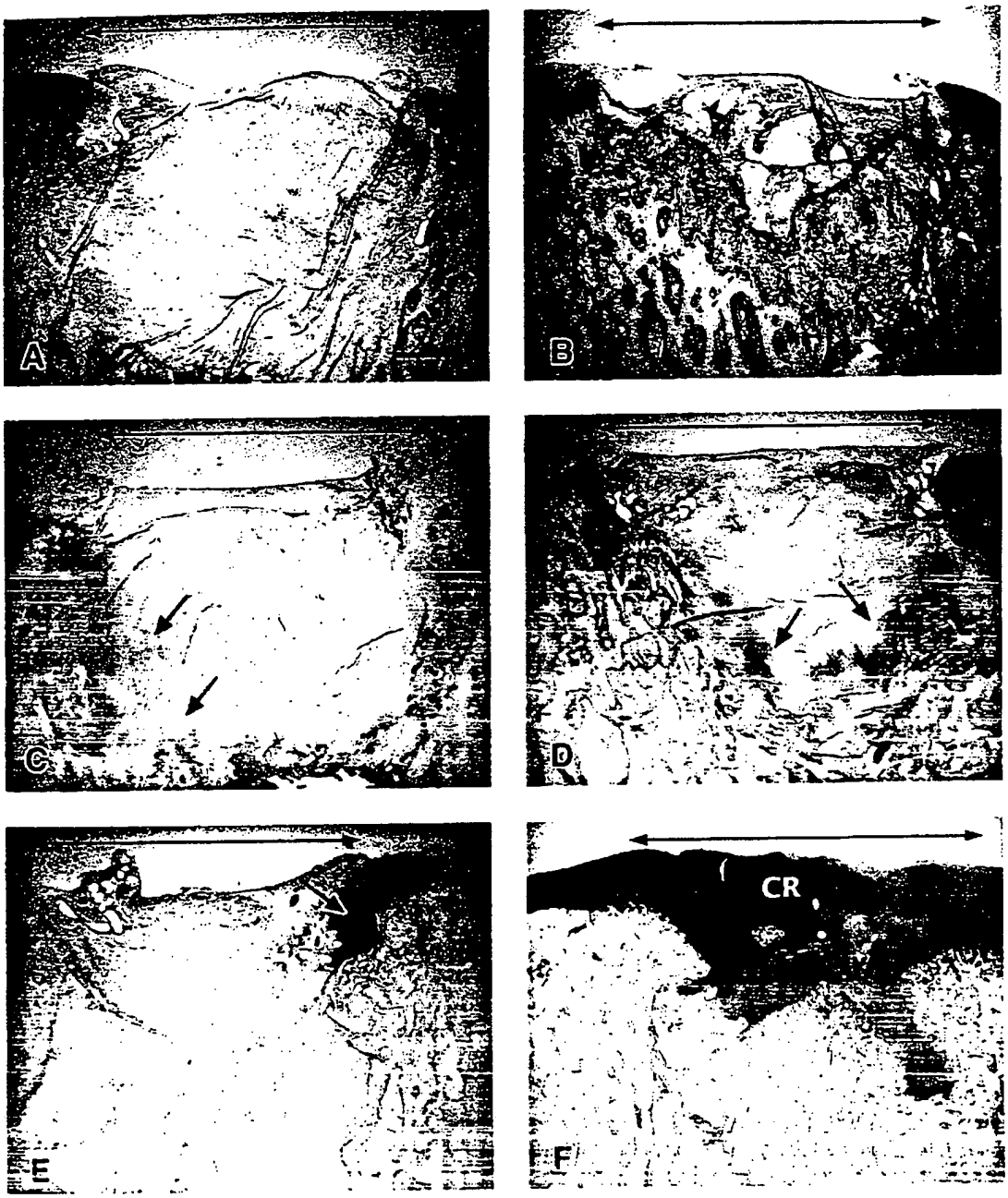

Figure 3. Overviews of defects treated for 1 month (A,B), 2 months (C,D) or 4 months (E, F) with rhOP-1 device (B,D,F) or without rhOP-1 (A,C,E). The bars with arrows indicate the width of the original defect. All defects also received fragments of morselized autologous ear perichondrium in the implants. After 1 month (A,B), the tissue filling the defect has become organized, but cartilage regeneration is not apparent. After 2 months (C,D), cartilage regeneration is apparent in the deeper part of the defect in both the control (C) and the OP-1-treated (D) defect (arrows). After 4 months (E,F), extensive cartilage regeneration (CR) has occurred in the OP-1-treated defect (F) but not in the control (E). Note that in the control defect (E), cartilage from the original defect wall seems to have moved into the defect (arrow). Undecalcified plastic sections are stained with toluidine blue. Bar in A is 2 mm.

with phosphate-buffered saline solution (PBS). The specimens for histology and biochemical analysis were prepared as described below.

Histology

For histology the defect halves, including subchondral bone, were fixed in 4% phosphate-buffered formalin and embedded, undecalcified, in polymethylmethacrylate (PMMA). Using a heavy duty Jung-K microtome with a steel knife, 3-μm thick sections were cut along the original plane of the defect bisectioning. Sections were stained with toluidine blue for examination of the metachromatic matrix or with Goldner's trichrome method for examination of bone formation. Representative sections from each specimen were selected. Examination was performed by two observers. The following parameters were assessed: tissue hyalinity (absence of coarse collagen fibers), metachromatic staining of the matrix with toluidine blue, surface irregularity, chondrocyte clustering, regenerated subchondral bone, bonding to the adjacent articular cartilage, inflammatory cell infiltration around the implant, and freedom from degenerative changes in the adjacent cartilage. Each of these characteristics was evaluated using a grading scale that was compiled out of grading scales used by other authors (Table II).[14–18]

Biochemical procedures

For biochemical analysis, control cartilage and tissue from the defect half were collected in ice cold PBS. The material was examined with a dissection microscope and divided into two parts, "superficial" and "deep," based on their macroscopical aspects (color and structure). Both samples were washed once with PBS and then rinsed in ice-cold water. They were lyophilized for 48 h to determine the dry weight. Then the samples were cut into 20-micrometer cryostat sections and lyophilized for another 24 h. Proteoglycans were extracted from the lyophilized sections with 60 mL/mg of dry weight of extraction buffer containing 4*M* of guanidinium HCl; 0.15*M* of potassium acetate, pH 5.8; and proteinase inhibitors (5 m*M* of benzamidinium HCl, 0.1*M* of 6-amino-n-hexanoic acid, 10 m*M* of EDTA, 5 m*M* of phenylmethylsulphonyl fluoride, and 5 m*M* of N-ethylmaleimide) at 4°C for 60 h. Supernatant and pellet were separated by centrifugation at 30,000 rpm for 30 min. The pellet was re-extracted with extraction buffer and centrifuged again. First and second supernatants were pooled, analyzed for proteoglycan content using dimethyl methylene blue,[19] and used for gel filtration. For gel filtration an aliquot (0.5 mL) of the supernatant was applied to a Sepharose CL-2B column (0.7 × 145 cm) and eluted with a buffer solution containing 4*M* of guanidinium-HCl, 0.1*M* of sodium sulphate, 0.05*M* of sodium acetate, and 0.1% triton X-100 at pH 6.1. The flow rate was 1.0 mL/h and 0.7-mL fractions were collected.[20] Fractions were analyzed for proteoglycan content based on dimethylmethylene blue binding.[19] The data were plotted as a Sepharose CL-2B profile (Fig. 2). The area under the first peak (Fig. 2, peak A) divided by the total area (Fig. 2, peaks A + B) represents the percentage aggrecan.

Statistics

The data were analyzed using the SPSS package (6.0 for Windows 95). From this package several subprograms for analysis of variance were used.

RESULTS

In two goats the surgical procedure was complicated by a small fracture of the lateral wall of the medial condyle. In one goat this did not lead to complications. In the other animal it led to infection of the defect and the animal was excluded (Table III; rhOP-1 device; 1 month implantation). Because the animal belonged to the 1-month group, and we found effects of OP-1 only after 4 months, we do not believe that this exclusion compromised the data. All goats recovered uneventfully from the anesthesia and started walking almost immediately. All the knee and ear wounds healed without problems. The limping decreased gradually, and at 4 weeks most animals walked normally.

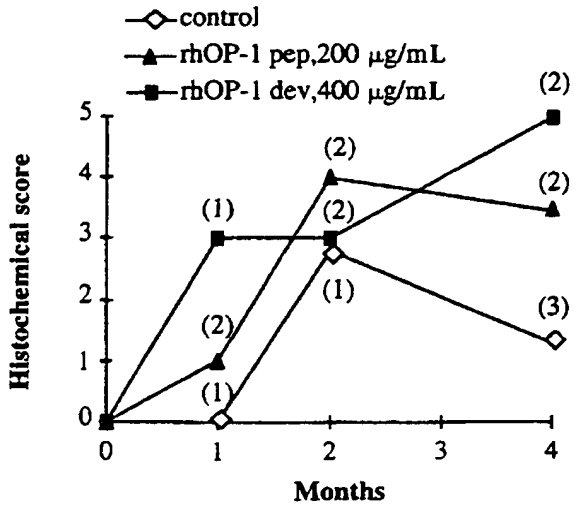

Figure 4. Histochemical score of the defects calculated as the sum of the scores for metachromasia and hyalinity, as shown in Table III. Control defects contained perichondrium only; rhOP-1 pep-rhOP-1 peptide: defects were treated with 200 μg/mL of rhOP-1 peptide, with or without perichondrium. The numbers in parentheses are the number of animals used. rhOP-1 dev-rhOP-1 device: defects were treated with 400 μg/mL of rhOP-1 device, with or without perichondrium.

At obduction, gross morphology of the operated joint, as scored with the macroscopic scoring system of Moran et al.,[14] showed no consistent differences between the animals treated with rhOP-1 and the control group; the mean score of all groups was five (highest score: 10; data not shown).

At histologic inspection, no systematic differences were apparent between the OP-1-treated and the control defects after 1 and 2 months (Table III; Fig. 3). However, at 4 months after implantation differences between OP-1 treated and control animals became apparent. In all 4 OP-1-treated defects, extensive cartilage formation had occurred while of the three controls, only one defect showed cartilage; the other 2 did not (Table III; Fig. 3). To study the cartilagenous quality of the tissue filling the defect in more detail, the sum of the two most important histologic indicators of cartilage quality, metachromasia and hyalinity, was calculated as a histochemical score. After 4 months, the histochemical score was approximately 2- to 3-fold higher in rhOP-1-treated goats than in controls (Fig. 4). At higher magnification (Fig. 5) the newly formed cartilage in the OP-1-treated defects showed a high cellularity, indicative of young cartilage [Fig. 5(B)]. Collagen fibers sometimes seemed to cross the border between old and new cartilage [Fig. 5(C,D)].

To verify these histologic results, we performed a detailed biochemical evaluation of the groups at 4 months after implantation. It was not possible to perform this analysis at earlier time points since the amount of aggrecan in the defects was below the analytical detection limit. After the dry weight of the superficial and deep part of the regenerated tissue was determined, proteoglycan content was determined, and the percentage of aggrecan analyzed using an analytic Sepharose CL-2B column (Fig. 6). Aggrecan content then was calculated as dry weight × proteoglycan content × percentage aggrecan (Table IV). Total aggrecan content was calculated as the sum of superficial and deep layer (Table IV). In all goats, the percentage of aggrecan was higher in the deeper layer than in the superficial layer (Table IV), suggesting that the quality of the regenerated cartilage was better in the deeper than in the superficial layer. An analysis of

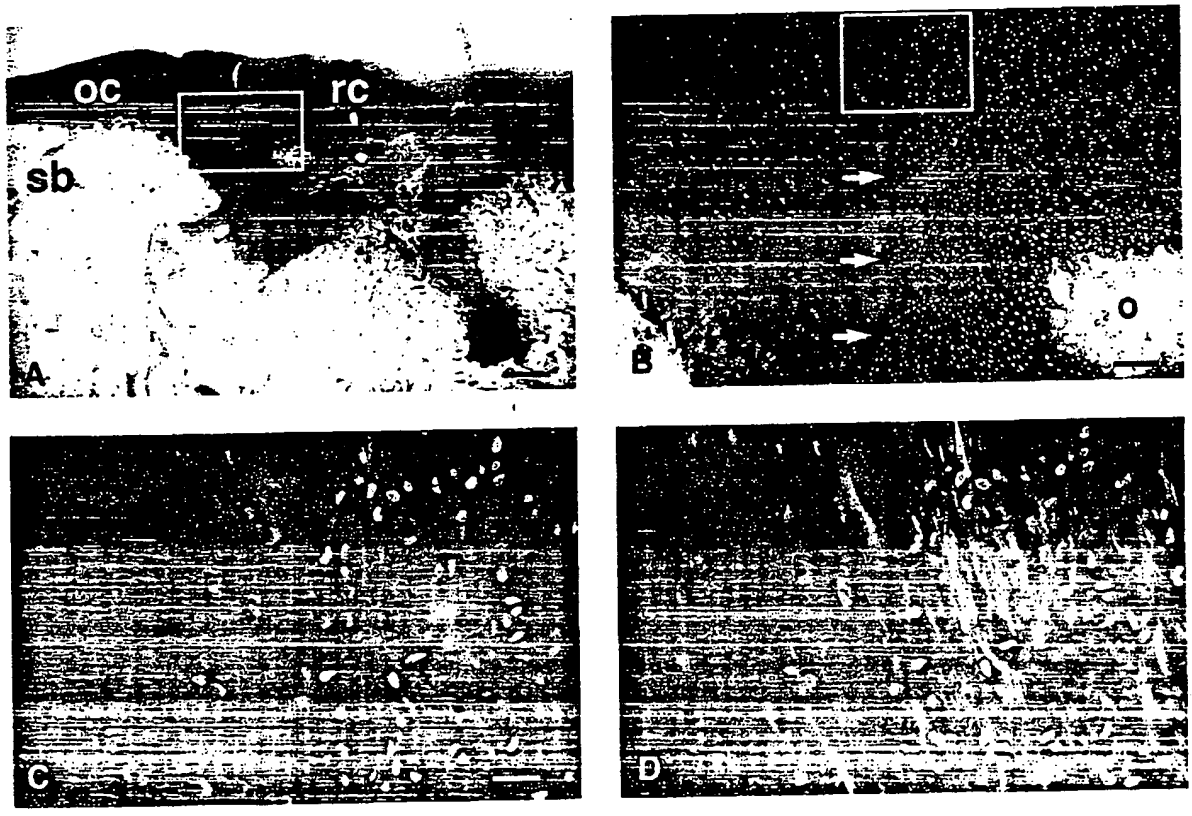

Figure 5. Details of defect treated with rhOP-1 device and perichondrium at 4 months after implantation. Undecalcified plastic section are stained with toluidine blue. (A) Overview of the defects: oc-original cartilage; rc-regenerated cartilage; sb-subchondral bone. The box indicates the enlarged area as shown in (B). (B) Detail of (A) at the junction of newly formed cartilage and residual articular cartilage. Small arrows indicate the border between the original cartilage (oc) and the regenerated cartilage (rc). o-center of ossification. The box indicates the area shown in (C). (C) Detail of (B), showing the close bonding between residual and newly formed cartilage. (D) The same detail viewed with polarized light shows continuity of collagen fibers between the two layers. Original magnification: (A) bar is 1 mm; (B) bar is 133 μm; (C,D) bar is 40 μm.

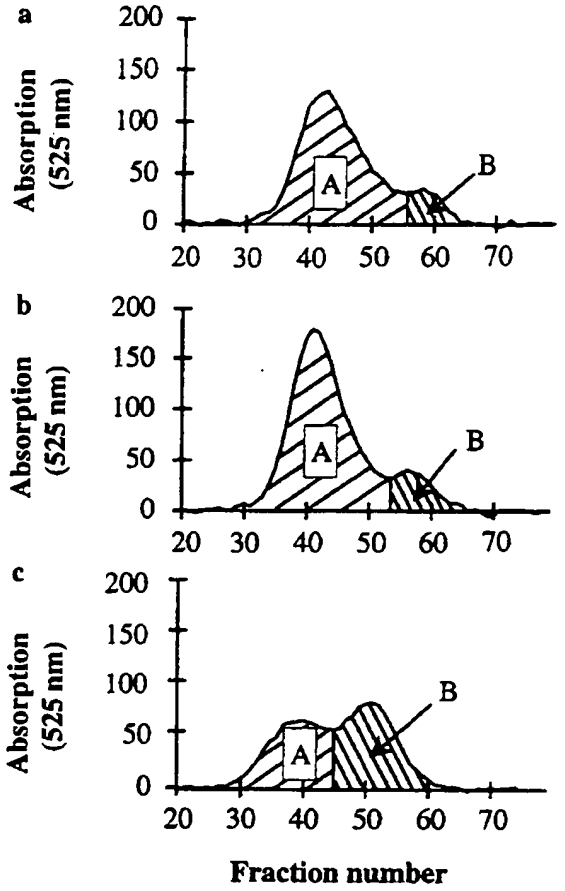

Figure 6. Elution profiles of sepharose CL-2B columns of three different 4-month-old defects: (a) normal articular cartilage; (b) defect treated with 400 μg/mL of rhOP-1 device and ear perichondrium; (c) defect treated with ear perichondrium only. In each profile, the percentage of aggrecan can be calculated as the area under peak A divided by total area (peaks A plus B).

variance was performed with aggrecan content as the dependent variable, OP-1 concentration (controls; 200 μg/mL; 400 μg/mL) as a between-subjects factor, and level (superficial, deep) as a within-subjects factor. None of the effects was significant. The aggrecan content main effect (i.e., the amount of aggrecan per defect level) came closest to significance, with $p = 0.056$. Because no effect of level was found, aggrecan content was summed over both levels and a one-way analysis of variance was performed, with this total as the dependent variable and rhOP-1 concentration as the between-subjets factor. Again, the aggrecan total content effect was close to significance, with $p = 0.056$. Furthermore there was a significant linear relationship between rhOP-1 concentration and total aggrecan content, $p = 0.026$ (Fig. 7). Deviation from linearity was not significant ($p = 0.59$).

To determine whether or not the histologic analysis agreed with the biochemical analysis, their correlation was calculated. A significant positive correlation was found between histochemical score and aggrecan content of the samples ($r = 0.893$; $p = 0.007$).

DISCUSSION AND CONCLUSIONS

As an experimental model of large articular defects, we used 9-mm wide subchondral defects in the articular cartilage of the medial condyle of the left knee in goats. Mature, fully grown goats were used to mimic the low regenerative capacity of adult articular cartilage in humans. The proportions of the knee joint of the goats made it possible to create a defect large enough so that it would not heal spontaneously, as is seen in small articular defects, for example in rabbits. The size of the goat condyle still allowed us enough cartilagenous border around the 9-mm defect for suturing of the periosteal flap, thereby mimicking the human situation. A similar operative procedure currently is followed in human trials.[21] Therefore this model permitted us to study the effect of rhOP-1 on cartilage formation in subchondral defects that mimic the human clinical situation.

The defects were filled with fresh autologous coagulated blood, mixed with rhOP-1 combined with collagen (rhOP-1 device) or rhOP-1 without collagen (rhOP-1 peptide). Since all the implants contained autologous blood, and because clotted blood always is present in surgical wounds, the implant mimicked the natural cellular environment during wound healing. The blood served as a medium to make implants of similar size and rhOP-1 content. Whether or not the blood clot also acted as a source of growth factors was not the subject of this study.

The dosages of rhOP-1 used were 200 μg/mL in the peptide group and 400 μg/mL in the device group. These dosages were based on earlier *in vitro* work of our group, in which rhOP-1 peptide at 40–400 μg/mL stimulated *de novo* formation of cartilage.[13] In this first *in vivo* experiment, we used the higher dosages.

In cartilage research, several extensive histologic scoring scales have been used.[15,16] Since the aim of this study was first to determine whether rhOP-1 is capable of stimulating hyaline cartilage formation in subchondral defects, we concentrated on the toluidine blue-staining of the matrix and the tissue hyalinity, that is, absence of coarse fibers in the Goldner-stained sections. These two parameters give the most valuable information on the cartilaginous properties of newly formed tissue. The biochemical analysis correlated well with the histologic findings and showed the appearance of proteoglycan-rich tissue in the rhOP-1-treated defects at 4 months. rhOP-1 increased the prevalence of aggrecan in both the superficial and the deep-layer in the 4-month defects, suggesting that rhOP-1 promoted the production of a cartilage-like matrix.

TABLE IV
Aggrecan Content of Defect Fillings at 4 Months After Implantation

Treatment	Defect Layer*	Mg Dry Weight	Proteoglycan Content†	Percentage Aggrecan‡	Aggrecan Content§
Control no. 1	superficial	9.6	11	26%	28
	deep	3.8	69	72%	183
	total	13.4			211
Control no. 2	superficial	9.2	9	15%	12
	deep	2.7	13	26%	9
	total	11.9			21
Control no. 3	superficial	9.8	33	48%	155
	deep	4.1	50	64%	131
	total	13.9			286
OP-1 pep‖ (no perich)	superficial	10.0	39	54%	289
	deep	4.1	77	68%	215
	total	14.1			504
OP-1 pep	superficial	10.8	28	49%	190
	deep	3.0	80	79%	148
	total	13.8			338
OP-1 dev** (no perich)	superficial	8.3	46	65%	248
	deep	5.1	63	77%	247
	total	13.4			495
OP-1 dev	superficial	8.1	50	67%	271
	deep	3.0	131	85%	334
	total	11.1			605

*The tissue filling the defect was divided into a superficial layer and a deep layer because of their different consistencies. †The total amount of proteoglycan as microgram per mg dry weight. ‡Percentage aggrecan of total proteoglycan content. §Aggrecan content, calculated as dry weight × proteoglycan content × percentage aggrecan. ‖OP-1 peptide was added at 200 μg/mL implant. **OP-1 device was added at 400 μg/mL implant.

It is well known that rhOP-1, as one of the BMPs, stimulates bone cell differentiation,[22,23] but an effect on chondroblastic differentiation only recently has been established.[12,13,24] rhOP-1 was shown to affect chondroblastic differentiation of osteoprogenitor cells derived from newborn rat calvariae.[24] In embryonic mouse bone rudiments in organ culture, rhOP-1 treatment induced the transition of perichondrium cells into chondrocytes and inhibited chondrocyte hypertrophy.[12] *In situ* hybridization showed that rhOP-1 treatment resulted in a shift of mRNA expression from type I collagen towards type II collagen in the perichondrium while proliferation of chondroblasts was enhanced.[25] A recent study on adult perichondrium in tissue culture demonstrated that short-time (24 h) exposure to rhOP-1 stimulated chondroblastic differentiation 3 weeks later.[13] These observations suggest that rhOP-1 can induce cartilage formation by promoting chondroblastic differentiation from immature stem cells. The results of the present *in vivo* study are in line with the earlier *in vitro* study, that is that rhOP-1 promotes hyaline cartilage formation in articular sites.

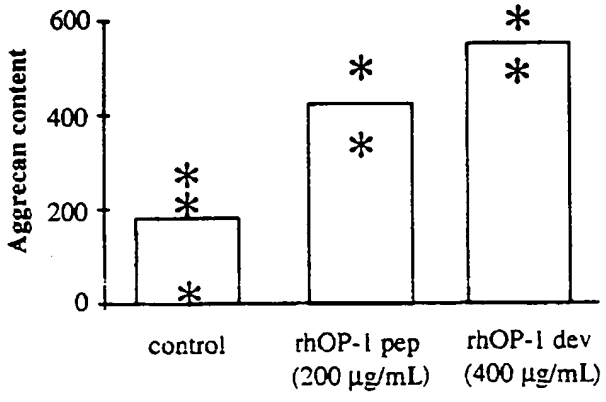

Figure 7. Aggrecan content, in μg per half defect, at 4 months after implantation. Individual values are indicated as asterisks while bars represent mean values. The relationship between rhOP-1 concentration and total amount of aggrecan was found significant in a one-way analysis of variance ($p = 0.026$).

We did not see large differences in the bonding of newly formed tissue to the adjacent cartilage between the control group and the rhOP-1-treated groups. However, the implication of the bonding in each group is different because the newly formed tissue was different between groups. Using polarized light microscopy, we found that collagen fiber orientation showed good bonding between old and new cartilage in the rhOP-1-treated defects, suggesting functional continuity between old and new cartilage and adaptation to prevalent loads. As good bonding of newly formed cartilage to the residual cartilage is difficult to obtain,[26–28] this observation underscores the potential of rhOP-1 for cartilage regeneration.

Addition of ear perichondrium gave no important further improvement of the effect of rhOP-1. Probably the supply of stem cells from the marrow stroma was

enough to make the addition of perichondrial stem cells unnecessary.

Recently Seller et al.[26] have demonstrated the effectiveness of rhBMP-2 in repairing defects of articular cartilage in rabbits. Although there are some differences in the protocol of their experiments and the present study, both studies show that cartilage repair is improved by the BMP growth factor. BMP-2 recently was reported to induce chondrogenic differentiation in multipotential murine C3H10T1/2 cells *in vitro*[29] while both BMP-2 and OP-1 (BMP-7) are closely involved with osteogenesis and chondrogenesis in the embryo,[24,30,31] including human embryos.[32] Although their specific functions in embryonic skeletogenesis are not yet clear, their actions for cartilage repair, alone or combined, deserve further study.

Successful results with articular cartilage regeneration have been reported by using cultured autologous chondrocyte transplantation.[21,27,33] However, recently Breinan et al.[34] demonstrated negative results with this method in a canine model. Therefore, since the current protocols for the repair of defects of articular cartilage produce variable results, a combination of chondrocyte transplantation and rhOP-1 treatment might offer an interesting alternative.

In sum, the present study suggests that osteogenic protein rhOP-1 stimulates cartilage formation in subchondral defects in goat knees at 4 months. Addition of perichondrial tissue did not seem to increase this effect. Further studies are warranted to establish the therapeutic potential of rhOP-1 for treating articular cartilage lesions.

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Contents

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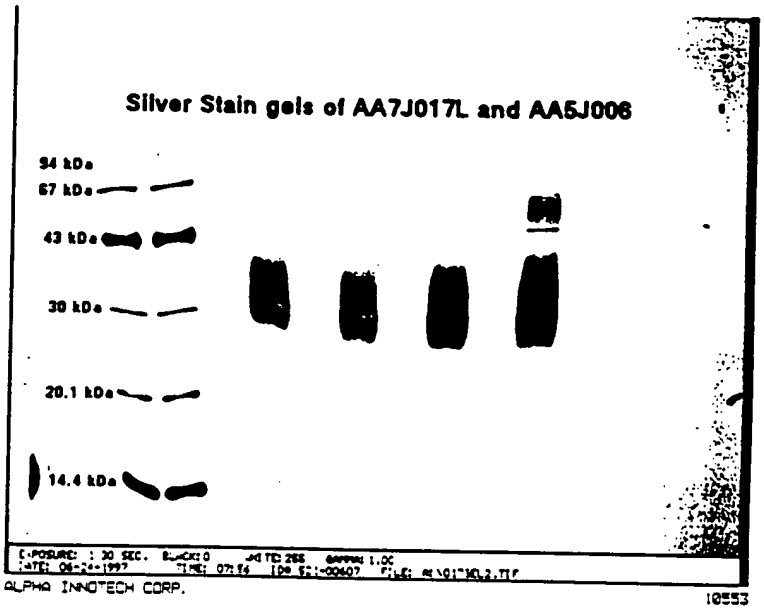
Cover: SEM photograph of cross-sectional interior of swollen (original magnification ×8,000) dextran-methacrylate hydrogels (DS = 0.09). Figure 9(B) from the article by Kim and Chu, pages 517–527, this issue.

Volume 49, Number 4 was mailed the week of December 27, 1999

Figure 5. F. 29b
SDS-PAGE Silver Stained Gel (Non-Reduced OP-1); Supplement 20 Lot AA5J006 and Commercial Lot AA7J017L



Lane	Sample	ng
1	Low MW Std	
2	Low MW Std	
3	Blank	
4	AA7J017L	500
5	Blank	
6	AA5J006	500
7	Blank	
8	AA7J017L	1000
9	Blank	
10	AA5J006	1000
11	Blank	
12	Blank	

A. 1 Overview of Volume 5, OP-1 Component

A flowchart of the major manufacturing process steps for NOVOS is illustrated in Figure 5. A. 1; process steps in the manufacture of OP-1 are enclosed in a box.

This process is further detailed in Figures 5. A. 2. through 5. A. 4.

Table 5. A. 1 summarizes the location of each manufacturing process, and the volume in which it is described.

Human Osteogenic Protein (OP-1) is a member of the TGF-ß (transforming growth factor-beta) superfamily. Like other members of this family, it is secreted from mammalian cells as a large dimeric precursor, referred to as Soluble OP-1. The Soluble OP-1 is comprised of two copies of the N-terminal Pro-domain and two copies of a highly conserved C-terminal domain.

The manufacture of OP-1 is performed at the CBM facility in West Lebanon, NH. Glycosylated soluble OP-1 is secreted into the culture medium in bioreactors by the recombinant production cell line.[Claim 3] The mature protein is obtained by specific elution from the first chromatography column, and is further purified by two additional chromatography columns. This volume describes the manufacture of OP-1, and a summary is provided below.

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Recombinant Human Osteogenic Protein-1 (hOP-1) Induces New Bone Formation *in Vivo* with a Specific Activity Comparable with Natural Bovine Osteogenic Protein and Stimulates Osteoblast Proliferation and Differentiation *in Vitro**

(Received for publication, April 30, 1992)

T. Kuber Sampath‡, James C. Maliakal, Peter V. Hauschka§¶, William K. Jones, Halina Sasak, Ronald F. Tucker, Kerry H. White, John E. Coughlin, Marjorie M. Tucker, Roy H. L. Pang, Clare Corbett, Engin Özkaynak, Hermann Oppermann, and David.C. Rueger

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We reported previously that a 32–36-kDa osteogenic protein purified from bovine bone matrix is composed of dimers of two members of the transforming growth factor (TGF)-β superfamily: the bovine equivalent of human osteogenic protein-1 (OP-1) and bone morphogenetic protein-2a, BMP-2a (BMP-2). In the present study, we produced the recombinant human OP-1 (hOP-1) in mammalian cells as a processed mature disulfide-linked homodimer with an apparent molecular weight of 36,000. Examination of hOP-1 in the rat subcutaneous bone induction model demonstrated that hOP-1 was capable of inducing new bone formation with a specific activity comparable with that exhibited by highly purified bovine osteogenic protein preparations. The half-maximal bone-inducing activity of hOP-1 in combination with a rat collagen matrix preparation was 50–100 ng/25 mg of matrix as determined by the calcium content of day 12 implants. Evaluation of hOP-1 effects on cell growth and collagen synthesis in rat osteoblast-enriched bone cell cultures showed that both cell proliferation and collagen synthesis were stimulated in a dose-dependent manner and increased 3-fold in response to 40 ng of hOP-1/ml. Examination of the expression of markers characteristic of the osteoblast phenotype showed that hOP-1 specifically stimulated the induction of alkaline phosphatase (4-fold increase at 40 ng of hOP-1/ml), parathyroid hormone-mediated intracellular cAMP production (4-fold increase at 40 ng of hOP-1/ml), and osteocalcin synthesis (5-fold increase at 25 ng of hOP-1/ml). In long-term (11–17 day) cultures of osteoblasts in the presence of β-glycerophosphate and L(+)-ascorbate, hOP-1 markedly increased the rate of mineralization as measured by the number of mineral nodules per well (20-fold increase at 20 ng of hOP-1/ml). Direct comparison of TGF-β1 and hOP-1 in these bone cell cultures indicated that, although both hOP-1 and TGF-β1 promoted cell proliferation and collagen synthesis, only hOP-1 was effective in specifically stimulating markers of the osteoblast phenotype.

Demineralized bone matrix when implanted in non-bony sites in rats induces a sequence of cellular events leading to the formation of new bone and bone marrow (1, 2). The finding that the bone-inducing activity elicited by demineralized bone matrix could be dissociated into a soluble component and assayed by implanting with an appropriate collagenous matrix carrier in rats (3) permitted the isolation of several osteogenic proteins (OPs),[1] also called bone morphogenetic proteins (BMPs) (4–7). The subsequent isolation of the genes encoding these proteins from human cDNA libraries identified a family of proteins, including BMP-2 through BMP-6, and osteogenic protein OP-1 (also referred to as BMP-7) (8–10). The predicted amino acid sequences of the proteins indicated that all are members of the TGF-β superfamily sharing a high degree of homology within the COOH-terminal 7 cysteine domain (11).

After purification and characterization, the 32–36-kDa bovine bone-derived osteogenic protein was found to be composed of disulfide-linked dimers of 18- and 16-kDa polypeptides (7); whether these exist exclusively as homodimers *in vivo* has not been established. Amino acid sequence data obtained from the proteolytically digested 18- and 16-kDa subunits indicate that the 18-kDa subunit is the bovine equivalent of mature human OP-1, whereas the 16-kDa subunit is the bovine equivalent of mature BMP-2. Recently, it was reported that homodimers of recombinant BMP-2 are capable of inducing bone when implanted with a collagenous matrix in rats (12) with a reported specific activity that was lower than that of naturally purified bovine bone inductive protein preparations.

A number of well known growth factors have been isolated and characterized from bone matrix extracts and from media conditioned by bone cells and bone organ cultures (13–19). They include: insulin-like growth factors I and II, transforming growth factors β1 and β2, acidic and basic fibroblast growth factors, platelet-derived growth factor, and hematopoietic factors. The synthesis and action of these local factors are in turn modulated by systemic factors, *e.g.* growth hor-

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[1] The abbreviations used are: OP, osteogenic protein; bOP, bovine osteogenic protein; hOP, human osteogenic protein; BMP, bone morphogenetic protein; TGF, transforming growth factor; PTH, parathyroid hormone; CHO, Chinese hamster ovary; HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MEM, minimum Eagle's medium; FBS, fetal bovine serum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IGF, insulin-like growth factor; FGF, fibroblast growth factor.

mone, parathyroid hormone, and vitamin D (19). These peptide growth factors are stored in bone and possibly released during bone remodeling. While they may play a critical role in the coupling of bone formation to bone resorption (21, 22), they have not been shown to induce new bone formation in the rat subcutaneous bone induction model (4). The newly identified family of osteogenic proteins isolated from bone matrix, on the other hand, are capable of inducing the commitment of stem cells to differentiate into osteoblastic cell lineages both *in vivo* (5–7) and *in vitro* (23–28). The action of these osteogenic proteins on bone cell cultures has been reported (23–28). Natural preparations of osteogenin (BMP-3), purified from bovine bone matrix, have been shown to stimulate chondrogenic and osteogenic phenotypes in culture (23, 24). Recombinant human BMP-2 induces the maturation of osteoblast precursor cells into osteoblast-like cells, although it has not been shown to have an effect on established osteoblast-like cells in culture (25–27). A recent study with recombinant BMP-4 (BMP-2b), a gene product closely related to BMP-2, showed that this protein is capable of stimulating DNA and collagen synthesis, as well as alkaline phosphatase activity in rat osteoblast-enriched cultures (28).

This report describes the characterization of purified recombinant hOP-1 and demonstrates that the recombinant human OP-1 is able to induce new bone formation in the rat subcutaneous assay with a specific activity that is comparable with highly purified bovine OP preparations and to promote cell proliferation and collagen synthesis of osteoblasts in culture. In addition, hOP-1 is shown to be effective in specifically stimulating markers characteristic of the osteoblast phenotype, *e.g.* alkaline phosphatase, osteocalcin, PTH-mediated cAMP, and deposition of mineralized nodules.

MATERIALS AND METHODS

Expression of hOP-1—The full-length hOP-1 cDNA was expressed in mammalian cells.[2] Briefly, the cDNA was inserted into a mammalian expression vector containing the amplifiable DHFR gene and stably transfected into dhfr(−) Chinese hamster ovary (CHO) cells. After methotrexate-mediated gene amplification, a cell line was selected, cultured in roller bottles or cell factories, and the conditioned media collected periodically. Alternatively, the cDNA was inserted into a mammalian expression vector containing the neomycin selective marker and the SV40 origin of replication and transfected into the BSC-1 ts58 cell line. BSC-1 is a modified monkey kidney-derived cell line that contains ts58, a temperature-sensitive mutant of the SV40 T-antigen (29). At 39 °C, the ts58 antigen is not active, whereas a temperature shift to 33 °C results in the expression of active T-antigen and the subsequent expression of appropriately transfected genes. The clones for BSC cells were selected by resistance to the drug G418 and subcultured.

Purification of hOP-1—During purification, the recombinant hOP-1 protein was detected by Western blot analysis using hOP-1 antisera. hOP-1 was purified from conditioned medium using three chromatography steps: S-Sepharose, phenyl-Sepharose (Pharmacia LKB Biotechnology Inc.), and reverse phase HPLC (C18 Vydac). A typical purification of hOP-1 utilized 2 liters of conditioned medium containing 0.5% fetal calf serum. The medium was diluted with 2 volumes of 9 M urea, 20 mM HEPES, pH 7.0 and applied to a 100-ml column of S-Sepharose equilibrated with 6 M urea, 20 mM HEPES, pH 7.0, containing 50 mM NaCl. After washing with equilibration buffer, step elution of bound protein was accomplished with the same buffer containing 100 and 300 mM NaCl. The 300 mM NaCl fraction was made 1.0 M in ammonium sulfate and applied to a 10-ml column of phenyl-Sepharose, pre-equilibrated with 6 M urea, 1.0 M ammonium sulfate, 0.3 M NaCl, 20 mM HEPES, pH 7.0. After washing with the equilibration buffer, the column was step-eluted with the same buffer containing 0.6 and 0 M ammonium sulfate. The protein eluted with 0 M ammonium sulfate was then sequentially dialyzed against water and 30% acetonitrile, 0.1% trifluoroacetic acid, and finally subjected to C18 reverse phase HPLC as described previously (7). Fractions containing hOP-1, as determined by immunoblot analysis and by Coomassie staining, were pooled. The purity of the hOP-1 was determined by gel scanning densitometry.

In Vivo Assay of hOP-1—Purified bovine OP (7), or recombinant human OP-1, in varying concentrations was combined with rat collagen carrier in 50% acetonitrile, 0.1% trifluoroacetic acid, lyophilized as described previously (7) and implanted in a subcutaneous site in the thorax region of 28–35-day-old male Long-Evans rats. Briefly, 25 mg of demineralized and 4 M guanidine HCl extracted rat bone matrix (rat collagen carrier) was added to osteogenic protein dissolved in 200 μl of 50% acetonitrile, 0.1% trifluoroacetic acid, mixed, and then lyophilized. The response exhibited by intact demineralized diaphyseal rat bone matrix (particle size 74–420 μm, 25 mg) was used as the maximal bone-forming activity and compared with the activity exhibited by bovine OP and recombinant hOP-1 containing implants. Rat bone collagen carrier was used alone as the negative control. The day of implantation was designated as day 0 of the assay. Implants were removed on days 5, 7, 9, 12, 14, and 21 for evaluation. Bone-forming activity in the implants was monitored by the specific activity of alkaline phosphatase, calcium content, and histological evaluation. The specific activity of alkaline phosphatase and the calcium content of the 0.5 M HCl-soluble fraction of the sediment were determined as described previously (2). For histological examination, implants were fixed in Bouin's Solution, embedded in JB4 plastic medium, cut into 1-μm sections, and stained by toluidine blue (American HistoLab, Gaithersburg, MD). The specific bone-forming activity was determined by analysis of the calcium content of day 12 implants and expressed as the amount of protein required to exhibit half maximal bone-forming activity, as compared with the bone-forming activity of the demineralized rat bone matrix implants.

Cell Cultures—Primary cultures of rat calvarial cells were obtained from 1–2-day-old CD and Long-Evans strain rats (Charles River Laboratories, Wilmington, MA) by sequential collagenase digestion essentially following the methods described by Wong and Cohn (30). Briefly, six sequential 20-min digests were performed on suture-free calvarial fragments at 37 °C in phosphate-buffered saline containing collagenase CLS-2 (2 mg/ml) (Worthington, Freehold, NJ). Single-cell suspensions were obtained at each digest interval and numbered as individual populations 1–6 and were pooled as populations 1–2, 3–5, and 6. The cells were plated in 24-multiwell plates (Falcon Labware, Lincoln Park, NJ) or 48-well tissue culture plates (Costar Corp, Cambridge, MA) at varying cell densities as described in the text in α-MEM (GIBCO) containing 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) with L-glutamine and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). After culturing at 37 °C, 5% CO_2, and 88% humidity for 3–5 days without changing the medium, and after achieving a density of approximately 5×10^4 cells/cm^2, the medium was replaced with serum-free medium for 24 h prior to the addition of hOP-1 or TGF-β1 (R & D Systems, Minneapolis, MN).

To evaluate the effect of hOP-1 on osteocalcin synthesis and the mineralization process, the medium was supplemented with 10% FBS. On day 2, cells were fed with fresh medium supplemented with fresh 10 mM β-glycerophosphate (Sigma). Beginning on day 5 and at twice weekly intervals, the cells were fed with a complete mineralization medium containing all of the above components plus fresh L(+)-ascorbate at a final concentration of 50 μg/ml. Purified hOP-1, stored at −20 °C in 50% acetonitrile (or 50% ethanol) containing 0.1% trifluoroacetic acid, was added to tissue culture wells directly at ≤5 μl/ml of medium with thorough mixing. Control wells received the solvent vehicle only. After refeeding, each conditioned medium sample was diluted 1:1 in radioimmunoassay buffer containing protease inhibitors (31), which was stored at −20 °C until assayed for osteocalcin.

Cell Growth—The effect of hOP-1 on osteoblast cultures was examined by determining the rate of [^{3}H]thymidine incorporation into total acid-insoluble DNA and cell number. DNA synthesis rates were determined in triplicate cultures after 24 h of hOP-1 treatment by adding [*methyl*-^{3}H]thymidine (2 μCi/ml, 80 Ci/mmol; Du Pont-New England Nuclear) for 6 h before the termination of the culture. Incorporation was terminated by aspiration of the medium, and after washing three times with phosphate-buffered saline, the trichloroacetic acid (10%)-precipitated radioactive DNA was extracted with 1.0% (w/v) sodium dodecyl sulfate, 0.1 M NaOH and quantitated by liquid scintillation counting. For cell number determination, 1×10^5 cells were plated in T-25 flasks (Falcon Labware, Lincoln Park, NJ)

[2] H. Oppermann, W. K. Jones, J. C. Maliakal, H. Sasak, E. Özkaynak, T. K. Sampath, D. C. Rueger, and R. H. L. Pang, unpublished data.

in α-MEM containing 10% FBS, and after 24 h the growth medium was replaced with serum-free medium containing various concentrations of hOP-1. For comparison, control cultures received fresh 10% FBS. Triplicate cultures were harvested every 24 h for the duration of 7 days, and the cell number was determined by counting of the cells released by trypsin digestion (GIBCO) in a fixed volume hemacytometer.

Collagen Synthesis—The rate of collagenous and noncollagenous protein synthesis was measured in osteoblast-enriched cultures by pulse labeling with 25 μCi/ml [2,4-^{3}H]proline (0.2–0.5 Ci/mmol, Du Pont-New England Nuclear) for the last 6 h of culture. Various concentrations of growth factors were added to confluent cultures (in triplicate) in 24-well plates containing serum-free medium. Following incubation, the cell layers were lysed by three freeze thaw cycles and extracted with 1 M NaCl, Tris-HCl buffer, pH 7.4, containing 10 mM *N*-ethylmaleimide, 250 nM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride. Proteins from both cell culture medium and cell lysates were precipitated with 10% trichloroacetic acid and chilled; pellets were washed with acetone:ether (3:1 v/v), dried, resolubilized in 0.5 M acetic acid, and neutralized with NaOH. The amount of [^{3}H]proline incorporated into collagenase-digestible protein and nondigestible noncollagenous protein was determined as described by Peterkofsky and Diegelmann (32). The percent collagen synthesis was calculated after correcting for the relative abundance of proline in collagenase-digestible protein (multiplying the values of noncollagenous proteins by 5.4).

Alkaline Phosphatase Activity—Alkaline phosphatase activity in cultured cells was determined by the method of Reddi and Huggins (2). Following removal of culture medium, cell layers were subjected to three freeze/thaw cycles and sonicated in 200 μl of assay buffer (0.15 M NaCl, 3 mM $NaHCO_3$, pH 7.4, containing 0.1% Triton X-100). Recovered samples (10 μl) were assayed for enzyme activity in 96-well plates with *p*-nitrophenyl phosphate (Sigma) as a substrate in glycine-NaOH buffer, pH 9.3, in a total volume of 100 μl; after 30 min at 37 °C the reaction was stopped with 100 μl of 0.1 M NaOH and absorbance was measured at 400 nm on a Dynatech MR700 plate reader with *p*-nitrophenol as a standard. Results are presented in units/μg of protein, where 1 unit = 1 nmol of *p*-nitrophenol liberated per 30 min at 37 °C.

3′,5′ cAMP Production in Response to PTH—To determine the cAMP production in the presence of PTH, cells were preincubated for 20 min with α-MEM containing 0.5% bovine serum albumin and 1 mM 3-isobutyl-1-methylxanthine (Sigma), and then 200 ng/ml of human PTH(1–34) (Sigma) was added and incubation continued for 8 min. The cell layers were solubilized in 0.1% Triton X-100, and the concentration of cAMP in the cell layer was determined using a cAMP assay kit (Amersham).

Osteocalcin Radioimmunoassay—Rat osteocalcin levels in the cell culture supernatant and cell-associated extracellular matrix were determined by a 3-day nonequilibrium radioimmunoassay as described previously (31), employing goat anti-rat osteocalcin (first antibody) and donkey anti-goat IgG (second antibody). Data are reported as nanograms of osteocalcin/ml of medium or as total nanograms of osteocalcin/culture. Osteocalcin associated with the extracellular matrix was detected by extracting washed cell layers with 0.5 ml of 0.5 M EDTA containing protease inhibitors (31).

Histochemical Analysis—Cell layers were rinsed with cold 0.9% NaCl, fixed in fresh 4% paraformaldehyde at 23 °C for 10 min, and stained for endogenous alkaline phosphatase at pH 9.5 for 10 min using the commercially available kit (Sigma). Purple-stained wells were then dehydrated with methanol and air-dried. Mineralization was determined by a modified von Kossa staining technique on fixed cell layers. After a 30-min incubation with 3% $AgNO_3$ in the dark, H_2O rinsed samples were exposed for 30 s to 254-nm UV light (Fotodyne) to develop the black silver-stained calcium phosphate nodules. Individual mineralized foci (≥20 μm) were counted under a dissecting microscope and expressed as nodules/culture.

hOP-1 Antibody—The cDNA clone that encodes the COOH-terminal conserved 7-cysteine region (TGF-β-like domain) of the hOP-1 gene (9) (amino acids 324–431, approximately 12.5 kDa) was expressed as fusion proteins in *Escherichia coli* (33). The OP-1 fusion proteins, which were produced intracellularly as inclusion bodies, were solubilized and cleaved using mild acid to release the leader peptide. Following renaturation and purification, the hOP-1 polypeptide was used to raise polyclonal antibodies in rabbits. Antisera were tested for reactivity to nonreduced and reduced bovine OP by immunoblot analysis.

Analytical Methods—Protein fractions were characterized by SDS-PAGE on 15% minigels (0.5 mm thick) with a 3% stacking gel (7). Samples dissolved in Laemmli sample buffer were heated in boiling water for 3 min with or without dithiothreitol (100 mM) prior to electrophoresis. For Western blot analysis, samples subjected to SDS-PAGE were transferred to immobilon (Millipore Corp.) and reacted with specific anti-OP-1 rabbit sera and subsequently with goat anti-rabbit linked to peroxidase. To examine the presence of carbohydrate on hOP-1, immobilon-transferred proteins were blotted with concanavalin A linked to peroxidase (34). Amino acid sequence analysis was performed using an Applied Biosystems Protein/Peptide Sequencer (model 470A) as described (7). hOP-1 concentration was determined by HPLC, based on the absorbance of the hOP-1 peak at 214 nm in reference to a known amount of a hOP-1 standard, which had been previously quantitated by amino acid analysis.

RESULTS

Recombinant hOP-1—The full-length hOP-1 cDNA clone encoding the hOP-1 precursor, including the signal sequence, was expressed in mammalian cells in order to obtain correctly processed and fully active protein. Cell clones expressing hOP-1 were selected on the basis of Northern hybridization and Western blot analysis, using hOP-1-specific probes and antisera. Purification of hOP-1 from either BSC- or CHO-conditioned medium yielded preparations of processed mature hOP-1 that were greater than 90% pure. Fig. 1 shows immunoblotted and Coomassie-stained aliquots of the purified preparations of hOP-1 after SDS-PAGE. The CHO hOP-1 showed four to five major dimer bands in the 34–38-kDa range, which migrated as two major bands of 19 and 17 kDa, as well as a minor band of 23 kDa upon reduction. The BSC hOP-1 migrated as a dimer of approximately 36 kDa, which after reduction migrated at approximately 18 kDa. In some preparations, the reduced CHO hOP-1 and BSC hOP-1 samples showed degradation products in varying amounts that migrated at 15–16 kDa (data not shown).

The hOP-1 precursor contains 431 amino acids and is approximately three times larger than the mature hOP-1 (Fig. 2*A*). The processing site for the mature protein was identified by NH_2-terminal amino acid sequencing of the mature hOP-1 and is located following arginine residue 292. Amino-terminal sequence analysis of the hOP-1 CHO-sourced 23-, 19-, and 17-kDa species and the BSC-sourced 18-kDa species demonstrated that each possessed the same NH_2-terminal sequence Ser-Thr-Gly-Ser... The COOH terminus of each species was intact as determined by *in situ* cyanogen bromide digestion and subsequent sequence analysis. Apparent degradation products migrating at approximately 15 kDa in some CHO hOP-1 preparations displayed four NH_2-terminal sequences corresponding to subunits with 119, 117, 116, and 114 amino acids of mature OP-1, whereas the 16-kDa degra-

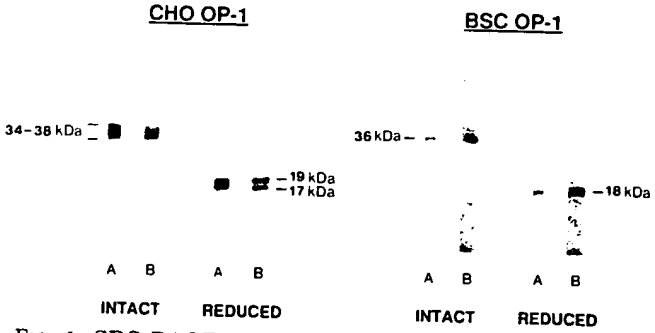

FIG. 1. **SDS-PAGE and immunoblot analysis of the purified mammalian cell produced human OP-1.** Lanes designated as *A* are immunoblots and lanes designated as *B* are Coomassie-stained. These OP-1 preparations were 90% pure, as determined by gel-scanning methods. Approximately 0.05 μg of OP-1 was used for immunoblots, and 0.5 μg of OP-1 was used for Coomassie staining.

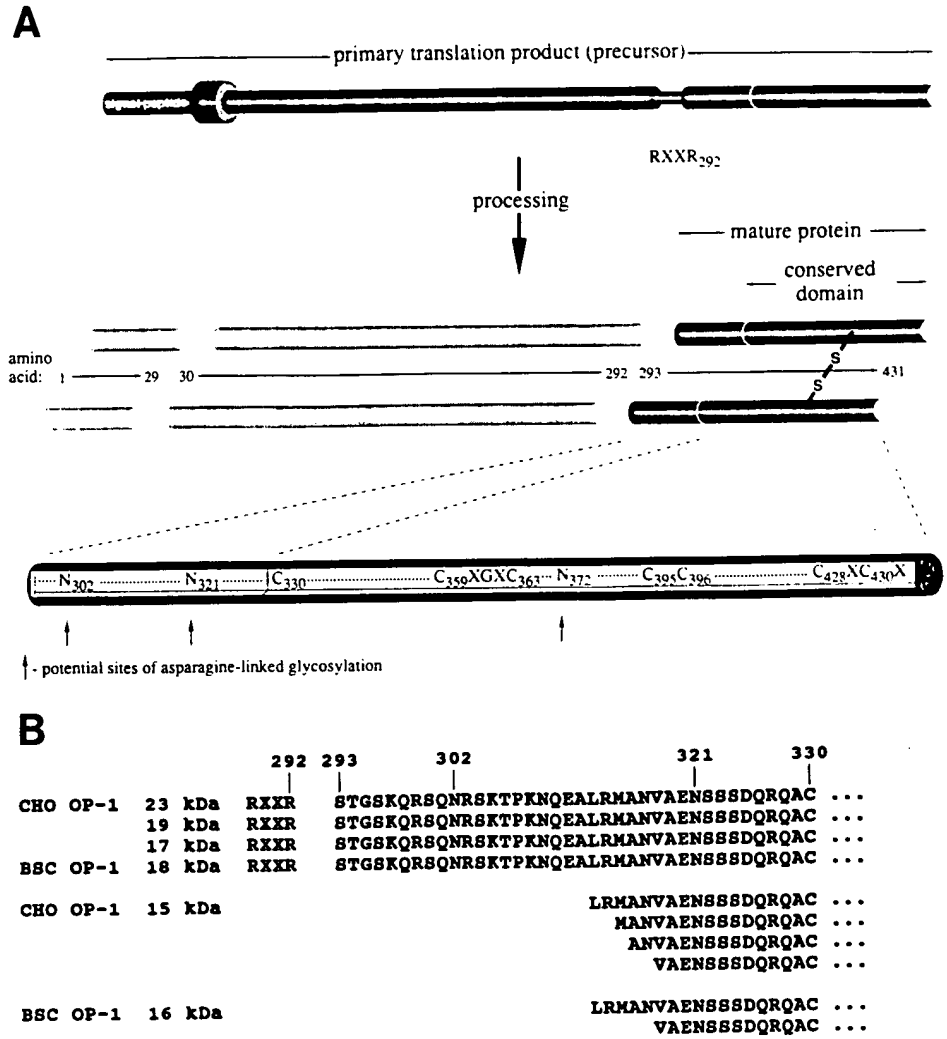

FIG. 2. *A*, structure of hOP-1. *B*, NH_2 terminus of CHO- and BSC-produced hOP-1 subunits.

dation product of BSC hOP-1 preparations presented two NH_2-terminal sequences corresponding to subunits with 116 and 114 amino acids (Fig. 2*B*).

Both the dimers and subunits of the mammalian expressed hOP-1 bound concanavalin A after SDS-PAGE and transfer to immobilon (data not shown). Identical NH_2-terminal sequences and the fact that the CHO hOP-1 and BSC hOP-1 subunits are reduced to a 14-kDa subunit after digestion with *N*-glycanase support the hypothesis that the apparent molecular weight differences of the CHO produced hOP-1 species are related to differences in glycosylation. Mature hOP-1 has three potential *N*-linked glycosylation sites at residues 302 (*N*-R-S), 321 (*N*-S-S), and 372 (*N*-S-T)). Two of the three potential glycosylation sites are within the NH_2-terminal region and the third is within the TGF-β domain (conserved 7 cysteine) region. Amino acid sequence analysis indicated that the glycosylation site at residue 372 in the TGF-β domain region is heavily or completely glycosylated, whereas the other two sites do not appear to be glycosylated to any measurable degree.

Bone-inducing Activity—The purified recombinant hOP-1 in combination with rat collagen as a carrier induced new bone formation *in vivo* as determined by histological examination of subcutaneous implants in the rat. Rat collagen carrier implants without hOP-1 did not show any sign of bone formation (Fig. 3*A*). In the rat subcutaneous implant assay, the bone collagen matrix carrier serves as a scaffold for the attachment and proliferation of mesenchymal cells which, in response to hOP-1, differentiate to form new bone at the implant site. In the absence of hOP-1 the collagen carrier implants contained mesenchymal cells and formed fibrous tissue which was slowly resorbed. On days 5–7, hOP-1 implants showed numerous chondrocytes in the implanted matrix (Fig. 3*B*). The degree of response was dependent on the dose of hOP-1 in that higher concentrations of protein elicited a more uniform response throughout the implant. Fig. 3*C* shows that day 9 implants exhibited calcification of cartilage and vascularization in the region of hypertrophied chondrocytes. Also, new bone formation was seen in apposition to calcified and cartilaginous matrix and basophilic osteoblasts surrounded the vascular endothelium. With higher doses of hOP-1, signs of remodeling were already apparent at day 9 as indicated by the presence of multinucleated osteoclasts. Although the implant predominantly induced endochondral bone, some intramembranous bone was seen at the outer surface of the implant (Fig. 3*D*). By day 12, chondrolysis was almost complete in that the implanted bone matrix was extensively resorbed and replaced by remodeled bone (Fig. 3*E*). Abundant osteocytes were seen surrounding the newly formed extracellular bone matrix (osteoid), and signs of hemopoiesis were apparent in the implant. Implants containing high doses of OP-1 showed evidence of further remodeling and recruitment of bone marrow elements. At 21 days, the carrier was almost completely resorbed and replaced with ossicles filled

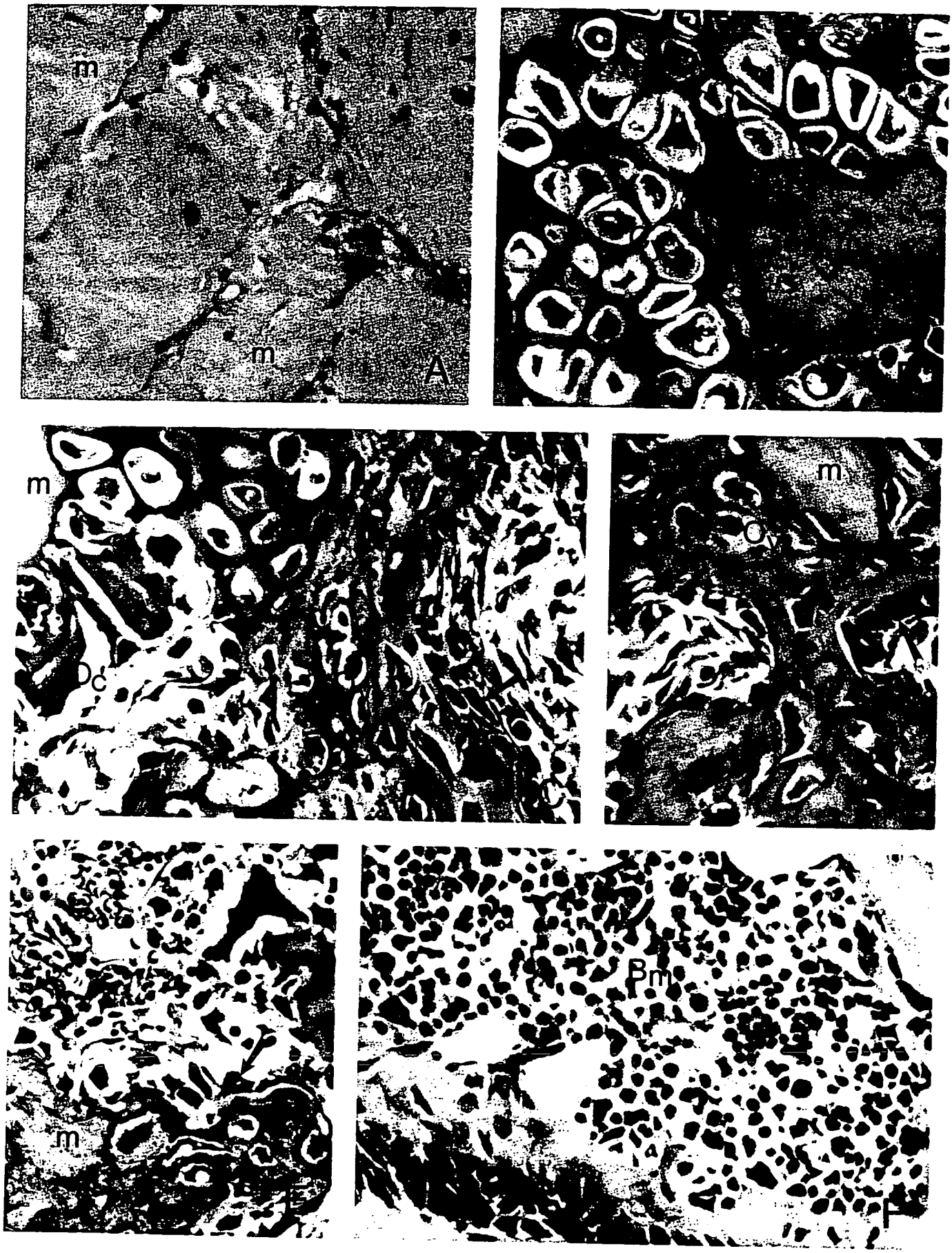

FIGURE 3.

with numerous bone marrow elements, including erythrocytic, granulocytic, and megakaryocytic cell lineages (Fig. 3F). Throughout the differentiation process the size of the newly formed bone was dependent upon the volume of the initial implant.

The bone-forming activity elicited by recombinant hOP-1 was monitored by alkaline phosphatase specific activity and quantitated by the calcium content of day 12 implants. Various quantities of hOP-1 protein (in nanograms) were used to develop a dose curve, and the hOP-1 bone-inducing activity was found to be reproducible and dose-dependent. Fig. 4A compares the specific activities of CHO and BSC cell-derived hOP-1 preparations to that of a highly purified natural bovine osteogenic protein preparation. The specific bone-forming activity of CHO hOP-1, as defined by the amount of protein required to exhibit half-maximal bone-inducing activity when compared with the activity of intact demineralized bone powder, was approximately 50–100 ng/25 mg of matrix implant. Control protein (mock) preparations from cells lacking the OP-1 expression vector obtained under identical conditions were not active. A broad range of doses including high concentrations of hOP-1 (0.025–50 μg/25 mg of matrix) were employed in order to evaluate the maximal bone-inducing activity of hOP-1 in the rat subcutaneous assay. Fig. 4B clearly illustrates the dependence of bone formation on the dose of hOP-1, as measured by the calcium content of day 12 implants. Bone formation plateaued at approximately 1 μg of hOP-1/25 mg of rat matrix. The bone-inducing activity exhibited by 1 μg of hOP-1/25 mg of matrix is approximately four times greater than that exhibited by the intact demineralized bone matrix. Evaluation of CHO hOP-1 preparations that contained predominantly the degraded dimer species (migrating at 15–16 kDa on SDS-PAGE under reducing condition) indicated that the bone-forming potential of these NH_2-terminally truncated forms of OP-1 were equivalent to that of intact mature OP-1 (data not shown).

Cell Proliferation—To evaluate the effect of hOP-1 on osteoblasts, we employed osteoblast-enriched primary cultures prepared through sequential collagenase digestions of newborn suture-free rat calvaria. Although these cultures have a heterogenous population of osteoblasts at various stages of differentiation (including preosteoblasts, lining cells, osteoblasts, and osteocytes), they are a standard model (30, 35) and are known to express phenotype characteristics of osteoblasts, including high levels of alkaline phosphatase, synthesis of type I collagen without type III collagen, secretion of osteocalcin into the medium, increased intracellular cAMP production in response to PTH, and the capacity to mineralize in long term culture (39–42). The osteoblast-enriched cultures used in our studies express these properties, and it is generally believed that these cultures are metabolically and functionally more representative of osteoblasts present in bone than are the established osteoblast-like osteosarcoma-derived cell lines.

The effect of hOP-1 on mitogenesis of osteoblast-enriched calvarial cells (preparations 3–5) was examined in the absence of serum using sparse, subconfluent, and confluent cultures and compared with the mitogenic response elicited by TGF-β1 and fresh fetal bovine serum (0.5–10%). The results showed that hOP-1 stimulated DNA synthesis in subconfluent and confluent cultures but not in sparse cultures, as examined by [^{3}H]thymidine incorporation into total acid-insoluble DNA (Fig. 5, A–C). Compared with control cultures, hOP-1 showed 3–5-fold maximal mitogenic stimulation at approximately 40 ng/ml in serum-free medium, whereas TGF-β1 showed a biphasic effect with 2–3-fold maximal stimulation between 0.01 and 10.0 ng/ml. Like hOP-1, TGF-β1 was effective in mediating mitogenesis in subconfluent and confluent cultures and showed no effect in sparse cultures (Fig. 5, A–C). The mitogenic activity of 40 ng of hOP-1/ml in serum-free medium was comparable with that elicited by 10% fetal calf serum alone (Fig. 1, B′ and C′).

The stimulatory effect of hOP-1 on the growth of osteoblasts was further examined by cell number measurements using subconfluent cultures in serum-free medium and compared with that elicited by 10% fetal calf serum. The growth of osteoblasts was stimulated by hOP-1 at a concentration of 80 ng/ml in serum-free medium as measured by the cell number at 24-h intervals over a period of 5 days (Fig. 6). At 5 days the growth-promoting activity of hOP-1 was approximately 80% of that produced by 10% fetal calf serum.

Collagen Synthesis—The effect of hOP-1 on collagen synthesis was examined using osteoblast-enriched cultures in serum-free medium and compared with the effect of TGF-β1. The results indicated that hOP-1 not only enhanced the absolute amount of collagen synthesized, as determined by the total incorporation of [^{3}H]proline into collagenase-digestible proteins, but also selectively increased collagen synthesis relative to the synthesis of noncollagenous proteins (Table I). The enhancement of collagen synthesis by hOP-1 was dose-dependent, exhibiting a maximum stimulation of 3-fold at a concentration of 40 ng/ml. TGF-β1 also promoted osteoblastic collagen synthesis in agreement with previously reported data and showed a maximum response at 5–10 ng/ml (36).

Osteoblast Phenotypic Markers—Effects of hOP-1 on osteoblast markers or activities known to be associated with their cellular functions were examined using osteoblast-enriched cultures and compared with TGF-β1 effects. The specific

FIG. 3. **Photomicrographs of the implants (stained with toluidine blue × 280) representing the various hOP-1-induced developmental stages of endochondral bone differentiation on days 7, 9, 12, and 21.** *A*, negative control (day 12), guanidine-extracted rat demineralized bone matrix (*m*) (rat carrier). Note the absence of new bone formation. The implant consists of bone matrix (*m*) and surrounding mesenchyme. *B*, rat carrier reconstituted with 125 ng of recombinant hOP-1 (day 7). Evidence of extensive chondrogenesis is seen. Newly formed cartilage cells, chondroblasts, and chondrocytes (*Cy*) are in close contact with the rat carrier matrix (*m*). *C* and *D*, rat carrier reconstituted with 250 ng of recombinant OP-1 (day 9). Note evidence of endochondral bone differentiation: *e.g.* cartilage calcification, hypertrophy of chondrocytes, vascular invasion, and the onset of new bone formation. Observe the appearance of basophilic osteoblasts (indicated by *arrows*) in close proximity to the vascular endothelium. Signs of remodeling are already apparent as indicated by the presence of multinucleated osteoclasts. Also, some intramembranous bone can be seen at the outer surface of the implant (*D*). *E*, rat carrier reconstituted with 125 ng of recombinant OP-1 (day 12), note the extensive bone formation and remodeling. The newly formed bone matrix deposited by the osteoblasts is being remodeled by multinucleated osteoclasts (*Oc*), and the implanted matrix is being slowly resorbed and replaced by remodeled bone. There are early signs of bone marrow recruitment in the newly formed ossicles. *F*, rat matrix reconstituted with 500 ng of recombinant OP-1 (day 21). Note hematopoietic bone marrow differentiation in the newly formed ossicles. Most of the implanted matrix (*m*) has been replaced by newly formed bone containing ossicles filled with bone marrow elements, including cells of erythrocytic, granulocytic, and megakaryocytic lineages.

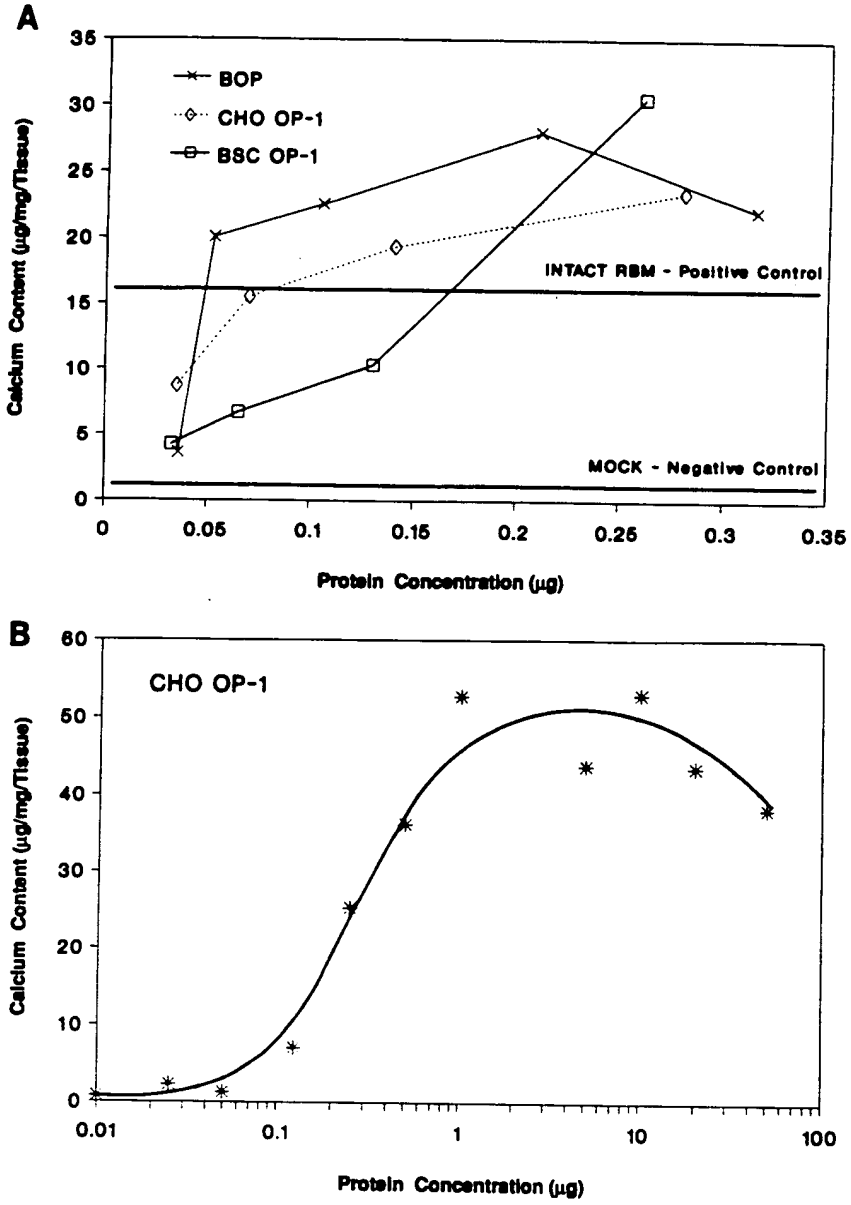

FIG. 4. *A*, comparison of bone-inducing activity exhibited by CHO- and BSC-produced recombinant hOP-1 with highly purified natural bovine OP. Calcium content in the day 12 implant was used to determine the extent of osteogenesis induced by increasing concentrations of hOP-1 or bovine OP in the implant. Bone-forming activity exhibited by the intact demineralized rat bone matrix was defined as maximal response and thus the half-maximal response is represented as 8 μg Ca^{2+}/mg of tissue. The rat carrier that contained a mock hOP-1 preparation was used as negative control. Values are average of six to ten observations from three to five rats. *B*, CHO hOP-1 dose curve. Bone-forming activity was quantitated by calcium content of the day 12 implant. The values are average of ten observations from five rats.

properties examined included: alkaline phosphatase activity (a marker enzyme for osteogenesis), PTH-mediated cAMP production (a marker for hormonal responsiveness of osteoblasts), synthesis of osteocalcin (a bone-specific protein and a marker for mature osteoblast activity and bone formation), and mineralization (a process characteristic of differentiated osteoblasts *in vivo* and of differentiated osteoblasts in long-term cultures containing ascorbate and β-glycerophosphate).

hOP-1 enhanced the specific activity of alkaline phosphatase in cell lysates of osteoblast-enriched cultures (Fig. 7, *B* and *C*). The effect of hOP-1 on alkaline phosphatase induction was dose-dependent and appeared to be specific to osteoblasts, since it did not stimulate alkaline phosphatase activity in non-osteoblastic cultures (Fig. 7*A*). Osteoblast-enriched cultures treated with 40 ng of hOP-1/ml for 3 days exhibited a 4-fold stimulation in the specific activity of alkaline phosphatase when compared with serum-depleted controls. In contrast, TGF-β1 did not increase the specific activity of alkaline phosphatase in osteoblast-enriched cultures and in fact diminished the enzyme activity at the higher concentrations tested. Examination of the effect of hOP-1 on the induction of alkaline phosphatase activity in osteoblast-enriched cultures over 6 days demonstrated a dose-dependent hOP-1 stimulation that reached a maximum at 48 h of incubation and remained at an elevated level thereafter (data not shown).

Table II shows that hOP-1 did not increase the level of intracellular cAMP in osteoblasts in the absence of PTH, suggesting that cAMP is not a second messenger for hOP-1. However, pretreatment with hOP-1 increased PTH-stimulated cAMP production in a dose-dependent manner; the addition of 200 ng of hOP-1/ml to osteoblast-enriched cultures in serum-depleted medium for 72 h produced a 6-fold increase in PTH-stimulated cAMP levels compared with control cultures not exposed to hOP-1. Under identical conditions to those tested with hOP-1, TGF-β1 did not affect PTH-mediated cAMP production.

Osteocalcin synthesis by osteoblast-enriched cultures was examined on days 3–21 by a specific radioimmunoassay. hOP-1 stimulated the induction of osteocalcin synthesis starting from day 7, reaching a peak on day 13 and then slowly declining (data not shown). Because of the long culture period required for the production of osteocalcin and subsequent mineralization, these studies were performed in the presence

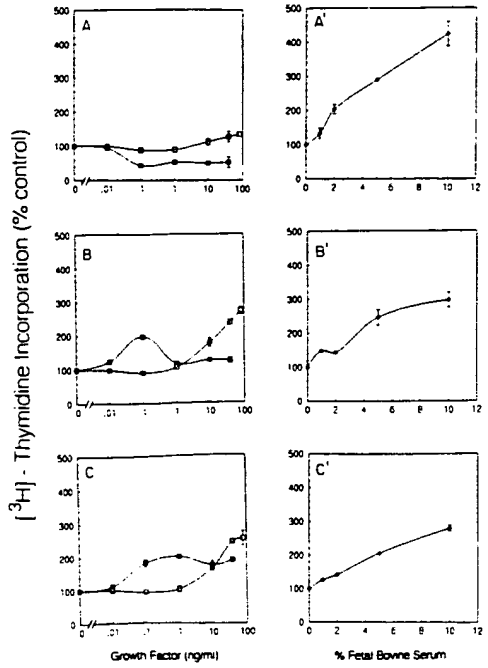

FIG. 5. **Effect of cell density on DNA synthesis by hOP-1 (□), TGF-β1 (■), and fetal calf serum (♦) in osteoblast-enriched cultures.** Population 3–5 cells were cultured in sparse (*A* and *A'*), subconfluent (*B* and *B'*), and confluent (*C* and *C'*) densities as described under "Materials and Methods." Cultures were incubated in serum-free medium for 24 h and then treated with either increasing concentrations of growth factors (*A–C*) or various percentage of fetal calf serum (*A'–C'*). After 18 h, the cultures were labeled with 2 μCi/ml [^{3}H]thymidine for 6 h. The incorporation of trichloroacetic acid-insoluble ^{3}H into cells was analyzed by solubilization of cells in SDS/NaOH and is represented as percent of serum-free medium control. Values are means ± S.E. of three cultures.

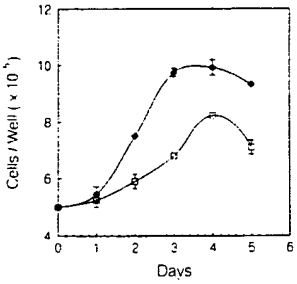

FIG. 6. **Effect of hOP-1 (□) and 10% serum (■) on cell numbers in osteoblast-enriched cultures.** Population 3–5 cells were plated at subconfluent density (10^5 cells/T-25 flask) in medium containing 10% FBS, and after 24 h the growth medium was replaced with serum-free medium containing hOP-1 (80 ng/ml). For comparison, control cultures received fresh 10% FBS. Triplicate cultures were harvested every 24 h, and the cell number was determined in trypsin-released single cell suspensions using a hemocytometer. Values are means ± S.E. from triplicate cultures.

of 10% serum. Fig. 8 shows the effect of varying concentrations of hOP-1 on osteocalcin production in day 13 cultures, as measured by radioimmunoassay. The hOP-1 effect produced approximately a 5-fold increase at 25 ng/ml compared with controls. The increase in osteocalcin synthesis correlated with increased mineralization in long-term osteoblast cultures as monitored by the appearance of mineral nodules (Fig. 9). hOP-1 increased the initial mineralization rate approximately 20-fold compared with untreated cultures. Evaluation of TGF-β1 effects on osteocalcin synthesis, and the rate of mineralization indicated that TGF-β1 neither induced osteocalcin

TABLE I

Influence of hOP-1 and TGF-β1 on collagen synthesis in osteoblast-enriched cultures

Cultures were grown to confluence, at which time the medium was replaced with serum free medium alone or containing growth factors. After 72 h [^{3}H]proline was added to the cultures for the final 6 h, and protein synthesis was measured (see "Materials and Methods"). Expressed as mean ± S.E. of triplicate cultures.

Concentration of protein		Collagenase-digestible protein	Noncollagenase protein	Collagen synthesis
hOP-1	TGF-β1			
ng/ml		*cpm × 10^{-3}*		%
		3.26 ± 0.06	4.36 ± 0.04	13.3
1		4.22 ± 0.05	4.86 ± 0.05	13.6
10		9.76 ± 0.12	7.32 ± 0.06	19.6
40		12.14 ± 0.10	8.02 ± 0.10	23.0
80		11.56 ± 0.08	6.50 ± 0.10	25.0
	1	8.51 ± 0.08	5.65 ± 0.05	21.0
	10	10.35 ± 0.16	5.26 ± 0.06	24.6
	40	11.45 ± 0.07	7.11 ± 0.10	23.3

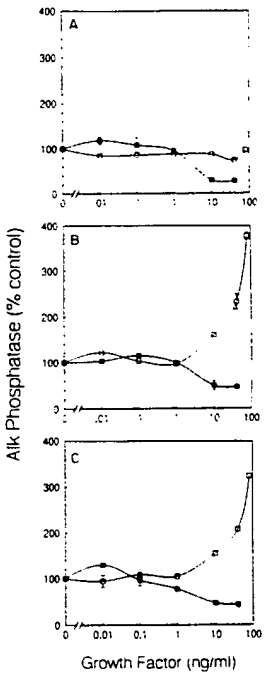

FIG. 7. **Effect of hOP-1 (□) and TGF-β1 (■) on stimulation of alkaline phosphatase (*AP*) activity in collagenase-released suture-free calvarial cells.** Cultures included: *A*, a pool of population 1 and 2 cells; *B*, a pool of population 3–5 cells; and *C*, population 6 cells. Confluent cultures in serum-free medium were treated with increasing concentrations of growth factors for 72 h. Cells were extracted with 0.15 M NaCl, 3 mM $NaHCO_3$, pH 7.4, and 0.1% Triton X-100 and incubated with substrate *p*-nitrophenyl phosphate for 30 min. The activity was determined by production of *p*-nitrophenol quantitated by absorbance at 400 nm. The specific activity of alkaline phosphatase (units/mg of protein) is presented as the percent of serum-free medium controls. Values are means ± S.E. of triplicate cultures.

synthesis nor promoted the mineralization process (data not shown).

DISCUSSION

We reported previously that the purified bovine osteogenic protein (bOP) preparations are composed of dimers of the bovine equivalents of OP-1 and BMP-2, two members of the TGF-β superfamily (7). Since previous data did not establish whether the bone-inducing activity of bovine OP is due to

TABLE II

Effect of hOP-1 on cAMP production in response to PTH in osteoblast-enriched cultures

Cultures treated with hOP-1 at various concentrations for 72 h were then washed and exposed to 0 (PTH(−)) or 200 ng/ml synthetic PTH(1-34) (PTH(+)) for 8 min in the presence of 1 mM 3-isobutyl-1-methylxanthine. Intracellular cAMP was extracted and measured by radioimmunoassay (see "Materials and Methods").

Growth factors	Amounts of cAMP produced		
	PTH(−)	PTH(+)	PTH(+)/PTH(−) ratio
ng/ml	*pmol/well*		
hOP-1			
0	1.87 ± 0.50	2.50 ± 0.03	1.3
1	2.15 ± 0.40	3.83 ± 0.57	1.7
10	1.50 ± 0.10	3.96 ± 0.35	2.6
40	2.05 ± 0.70	7.50 ± 0.50	3.6
100	1.55 ± 0.05	8.50 ± 1.40	5.4
200	1.90 ± 0.30	12.00 ± 0.70	6.3
1000	1.43 ± 0.05	14.75 ± 1.06	10.3
TGF-β1			
0	1.01 ± 0.07	2.26 ± 0.28	2.2
0.1	0.92 ± 0.15	1.38 ± 0.22	1.5
1	0.82 ± 0.09	1.25 ± 0.15	1.5
5	0.71 ± 0.32	0.90 ± 0.05	1.2

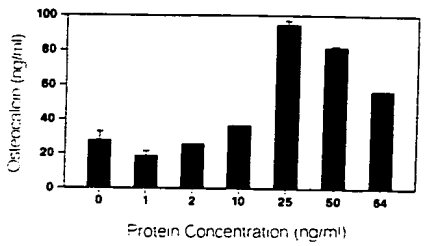

FIG. 8. **Effect of hOP-1 on osteocalcin synthesis in osteoblast-enriched cultures.** Population 3–5 cells in confluence were cultivated in medium containing 10% FBS. Beginning on day 5 cells were supplemented twice a week with fresh 10 mM β-glycerophosphate and L(+)-ascorbate (see "Materials and Methods"). Varying concentrations of hOP-1 were added to the cultures at day 5 and at every feeding. Control cultures received equal volumes of the hOP-1 solvent vehicle. Osteocalcin in the medium on day 13 was measured by radioimmunoassay and represented as nanograms/ml culture medium. Values are means ± S.E. of triplicate cultures.

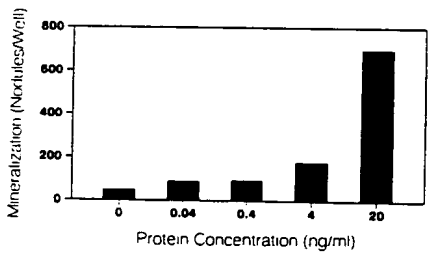

FIG. 9. **Effect of hOP-1 on the mineralization of osteoblast-enriched cultures.** Population 3–5 cells were cultured to confluence in the presence of 10% FBS and supplemented with 10 mM β-glycerophosphate and 50 μg of L(+)-ascorbate as in Fig. 8. On day 11, cells were rinsed with cold 0.9% NaCl, fixed in fresh 4% paraformaldehyde at 23 °C for 10 min and stained with 3% $AgNO_3$ (von Kossa Staining). Individual mineralized foci (≥20 μm) were counted in triplicate wells and represented as nodules/well. Three independent experiments yielded similar results.

homodimers and/or heterodimers of OP-1 and BMP-2, we produced recombinant hOP-1 to demonstrate that the homodimer of this polypeptide is capable of inducing new bone formation *in vivo* with a specific activity that is comparable with that of naturally sourced bovine OP. Additional studies have demonstrated that pure recombinant hOP-1 promotes the growth of osteoblasts and stimulates expression of markers characteristic of the osteoblast phenotype in culture.

This present study describes the purification and characterization of recombinant hOP-1 homodimers produced in mammalian cells (CHO and BSC). As predicted by analogy to the other members of the TGF-β superfamily, the hOP-1 gene is produced as a processed mature disulfide-linked homodimer as determined by 1) NH_2-terminal amino acid sequence analysis, 2) Western blot analysis with OP-1 antisera, and 3) SDS-PAGE analyses under nonreducing and reducing conditions. The NH_2-terminal amino acid sequences obtained from the purified hOP-1 subunit indicate that the cleavage site in the hOP-1 precursor occurs following the sequence, arginine-*X*-*X*-arginine, which is a consensus processing site for other members of TGF-β superfamily and a number of prohormones (37). The correctly processed mature hOP-1 subunit contains 139 amino acids. The mammalian-expressed hOP-1 is glycosylated as shown by its ability to bind concanavalin A and wheat germ agglutinin lectins. Studies on the CHO cell-produced protein show that hOP-1 can exhibit heterogeneity due to differential glycosylation.

The recombinant hOP-1 induces new bone formation *in vivo* in a manner that is highly reproducible and dose-dependent, irrespective of the cell used for production of hOP-1. Histological evaluation showed that as little as 5–10 ng of hOP-1 in 25 mg of matrix carrier is sufficient to induce endochondral bone formation in day 12 implants. In the absence of hOP-1, the collagen carrier induced formation of fibrous tissue that was eventually resorbed. The specific activity of hOP-1, defined as the amount of protein required to exhibit half-maximal bone-forming activity when compared with the activity of intact demineralized bone powder, is approximately 50–100 ng/25 mg of matrix implant (Fig. 4, *A* and *B*). This activity is comparable with that exhibited by the purified natural bOP. The sequence of cellular events leading to endochondral bone formation that occurs in response to rat matrix implants containing recombinant hOP-1 is identical to that induced by purified natural bOP and intact demineralized bone matrix. A uniform response is observed throughout the implant site during the developmental stages of cartilage, bone, and bone marrow differentiation. Histological evaluation showed that in implants containing higher amounts of hOP-1 (more than 1 μg of hOP-1/25 mg of matrix) bone development, *viz.* onset of bone formation, bone remodeling, and hematopoiesis, occurs 3–5 days earlier than when elicited by the rat demineralized bone matrix implants. Furthermore, implants containing higher amounts of hOP-1 showed that the matrix carrier is almost resorbed by 12 days after implantation and has become filled with newly formed bone and bone marrow elements. In contrast, implants of intact demineralized matrix show complete resorption of matrix by 18–21 days after implantation (2).

The quantitation of bone formation by calcium content of implants on day 12 after implantation demonstrates that the quantity and rate of bone formation is dependent upon the amount of hOP-1. Thus, the rate of bone formation can be modulated to levels less than, equivalent to, or greater than that exhibited by intact demineralized rat bone matrix by varying the amount of hOP-1 in the implant. The bone-forming activity, however, plateaus at a hOP-1 concentration of 1 μg/25 mg of matrix carrier. At this concentration of hOP-1 the bone-forming activity of the implant is four times greater than the activity elicited by intact demineralized bone matrix.

Homodimers of recombinant hBMP-2 have been reported to induce bone formation when implanted with rat collagen

carrier in the rat subcutaneous assay model (12). In these studies, approximately 10 times more rhBMP-2 was required to achieve the same level of bone-forming activity as that observed with the corresponding naturally sourced bovine bone inductive protein preparations (5, 12). Although we have not directly compared the activities of recombinant hOP-1 and hBMP-2, the present study demonstrates that the specific bone-inducing activity of hOP-1 is comparable with the naturally sourced bovine OP in the rat subcutaneous model.

Comparison of amino acid sequences of the TGF-β domains demonstrates that the other members of the TGF-β superfamily exhibit the following degrees of identity to human OP-1: BMP-5 (90%), murine Vgr-1 (BMP-6) (88%), *Drosophila melanogaster* 60A (70%), BMP-2 (60%), BMP-4 (58%), *D. melanogaster* DPP (58%), *Xenopus laevis* Vg-1 (57%), growth and differentiation factor, GDF-1 (42%), inhibin βA (44%), BMP-3 (42%), inhibin βB (39%), TGF-β1 (34%), and Müllerian inhibiting substance, MIS (32%) (8–10, 38). The homology in the conserved 7-cysteine domain of the TGF-β superfamily members suggests that some of these members may have *in vivo* osteogenic activity. Hammonds *et al.* (22) have recently reported that homodimers of recombinant hBMP-4, which is closely related to BMP-2, is also capable of inducing bone *in vivo*, whereas recombinant activin does not. TGF-β1 does not induce new bone formation under the same assay conditions (4, 22). Evaluation of the bone formation potential of other TGF-β superfamily members will provide valuable insight into the functional relationships of this family of proteins.

In cell culture studies, hOP-1 stimulates osteoblast proliferation which is dependent on both the concentration of hOP-1 and cell density. The mitogenic response elicited by hOP-1 in serum-free medium is comparable with the response obtained by adding fresh 10% FBS. The mitogenic effect of hOP-1 observed for the calvarial cells released initially following treatment with collagenase suggests that these mesenchymal cells (preosteoblasts) are also capable of responding to hOP-1 and may have hOP-1 binding sites or surface receptors (data not shown). Natural preparations of osteogenin (BMP-3) from bovine bone (23, 24) and recombinant BMP-2 (25–27) have been shown to stimulate the growth of preosteoblasts, periosteal cells, and C26 clonal rat osteoblast progenitors and to inhibit the proliferation of established osteoblast-like cells, MC3T3-E1 cells, and C20 clonal rat osteoblast cells. Recombinant human BMP-4, a gene product closely related to BMP-2, has been shown recently to stimulate DNA synthesis in rat osteoblast-enriched cultures (28). Our results show that recombinant hOP-1 stimulates the proliferation of both preosteoblasts and mature osteoblasts in culture.

Recombinant hOP-1 enhances the synthesis of collagenase-digestible protein and increases the percent collagen synthesis relative to that of noncollagenous proteins when added to osteoblast-enriched cultures in serum-depleted medium. Recombinant BMP-2, on the other hand, has been reported to have no effect on the expression of type I collagen mRNA or collagen synthesis by osteoblast-like cells, calvarial cells, and embryonic fibroblasts in culture (27). Osteogenin purified from bovine bone has been reported to stimulate the synthesis of collagenase-digestible protein by calvarial osteoblasts in culture (24). It is possible that a portion of this response can be attributed to small amounts of OP-1 or related proteins present in naturally sourced preparations of osteogenin (6). Recently, BMP-4 has been shown to enhance Type I collagen synthesis in osteoblast-enriched cultures (28).

The specific activity of alkaline phosphatase, the production of cAMP in response to PTH, osteocalcin synthesis, and extracellular matrix mineralization are all enhanced in osteoblast-enriched cultures in response to recombinant hOP-1. The effect of hOP-1 on alkaline phosphatase induction appears to be specific to osteoblasts, since it was not observed with non-osteoblastic cultures of population 1 and 2 rat calvarial cells and NIH-3T3 fibroblasts. hOP-1-induced alkaline phosphatase activity remained elevated over the entire period after the hOP-1 exposure. Recombinant BMP-2, BMP-4, and natural preparations of osteogenin have also been shown to stimulate alkaline phosphatase in calvarial osteoblasts, MC3T3-E1 cells, periosteal cells, and mouse 10T1/2 cells (23–28). With respect to hOP-1 regulation of other markers of the osteoblast phenotype, the production of cAMP in response to PTH is not only maintained by the presence of hOP-1 but also increases with increasing hOP-1 in these cultures. Adenylate cyclase activity which is subject to PTH stimulation is a biochemical marker of the mature osteoblastic phenotype (30), and hOP-1 increases this activity. Our study shows that hOP-1 is also capable of stimulating osteocalcin synthesis in osteoblast-enriched cultures without the addition of 1,25-$(OH)_2$ vitamin D_3. Osteocalcin, a bone-specific protein, is known to be synthesized by mature rat and human primary osteoblasts in long-term culture (39, 42). Furthermore, the hOP-1 induction of osteocalcin synthesis correlates with an increase in osteocalcin mRNA levels (data not shown). Vitamin D is known to up-regulate osteocalcin mRNA expression and osteocalcin synthesis when added to cultured osteoblasts (19, 43–45). BMP-2 has been shown to stimulate osteocalcin synthesis when added to osteoblast precursors in the presence of vitamin D *in vitro* (27). The hOP-1-induced osteocalcin synthesis, as evaluated here by radioimmunoassay and Northern blot mRNA analysis, appears to correlate with increased mineralization. Although osteoblast-enriched cultures used in these studies are capable of mineralizing in long term culture without addition of hOP-1, the initial rate at which mineralized foci appear can be enhanced at least 20-fold in the presence of hOP-1.

As hOP-1 is a member of the TGF-β superfamily, we evaluated the direct effect of TGF-β1 on calvarial-derived cell populations under identical conditions. Like hOP-1, TGF-β1 stimulates the proliferation of calvarial cells and synthesis of collagenase-digestible proteins in osteoblast-enriched cultures. TGF-β1, known to be synthesized by osteoblasts as an autocrine factor (36, 46), also exhibits biphasic effects on osteoblast proliferation and is reported to enhance collagen synthesis by osteoblasts *in vitro* (36). However, unlike hOP-1, TGF-β1 does not enhance the expression of markers characteristic of the osteoblast phenotype. In fact, significant decreases are caused by TGF-β1 in alkaline phosphatase specific activity (Fig. 5), PTH-mediated cAMP production (Table II), and osteocalcin synthesis (47) in osteoblast-enriched cultures. Other peptide growth factors that are known to stimulate bone cell proliferation *in vitro* include IGF-I, IGF-II, TGF-β2, TGF-β3, and acidic and basic FGF (20, 21). IGF-I and TGF-β1 have also been shown to enhance the collagen synthesis by calvarial osteoblasts in culture (36, 48).

In general, the concentrations of hOP-1 which exhibit biological activity *in vitro* appear to be higher than the concentrations typically employed for other cytokines and growth factors in similar experiments. The effective dose range for hOP-1 in the assays performed in our study is 1–40 ng/ml. In comparison the effective dose for TGF-β1 is in the range of 0.01–10 ng/ml. Results from *in vitro* studies have recently been reported for BMP-2 (25–27). BMP-2 enhancement of cell proliferation and induction of alkaline phosphatase in C26 clonal osteoblast precursor cells occurs at doses of 10–1000 ng/ml (27). BMP-4, on the other hand, has been reported

to enhance DNA and collagen synthesis of osteoblast-enriched cultures at doses of 0.1–10 ng/ml (28). These differences in dose response suggest that hOP-1 and related bone morphogenetic proteins may regulate cellular processes through cross-talk at their respective receptors and/or may exhibit differential binding affinity for a common receptor site at the cell surface. Alternatively, there could be specific binding proteins/carrier proteins synthesized by responding cell types which interact with exogenously added OPs or BMPs and thus modulate the specific activity locally. Differences in the solubility properties observed among the members of the TGF-β superfamily further complicate comparisons of dose dependence; TGF-β1 and BMP-4 are soluble in phosphate-buffered saline or 4 mM HCl containing 1% bovine serum albumin (49), whereas hOP-1 is not soluble under similar conditions. hOP-1 is also observed to adhere to plastic culture surfaces and to resist removal by serum-containing media. Evaluation of hOP-1 and related BMPs in various assay systems under identical conditions, and further identification of their specific cell surface binding sites (receptors), would significantly contribute to the understanding of differences in the dose response characteristics for these factors *in vitro* and *in vivo*.

In summary, these studies demonstrate that recombinant hOP-1, combined with a collagen matrix preparation and implanted subcutaneously in rats, induces new bone formation with a specific activity comparable with natural bovine OP. Studies in rabbits have shown that implantation of the hOP-1-containing matrix preparation into surgically created large diaphyseal segmental defects leads to the regeneration of new bone which is fully functional, both biologically and mechanically (50). The availability of recombinant hOP-1 in large quantities will permit the evaluation of its therapeutic potential in orthopedic medicine, certain types of plastic surgery, and various periodontal and craniofacial reconstructive procedures. Furthermore, the finding that hOP-1 promotes the proliferation of osteoblasts *in vitro* and causes specific increases in phenotypic markers known to be associated with the bone-forming functions of mature osteoblasts suggests that hOP-1 may play a significant role in the regulation of normal skeletal development, as well as in the control of bone remodeling in various metabolic bone diseases.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES Public Health Service

Food and Drug Administration
Center for Devices and
Radiological Health
1390 Piccard Drive
Rockville, Maryland 20850

August 4, 1991

STRYKER CORP.
2725 FAIRFIELD ROAD
KALAMAZOO, MI 49002
ATTN: SAMUEL YIN, PH.D.

Dear Sponsor:

The information you have submitted, as required by the Food and Drug Administration (FDA) investigational device exemptions (IDE) regulation, has been assigned the following document control number:

IDE Number: G910130
Dated: 08/01/91
Received: 08/02/91
Device: STRYKER OSTEOGENIC PROTEIN (OP) DEVICE

FDA will notify you when the review of this submission has been completed or if any additional information is required. In accordance with Section 812.30 of the IDE regulation, you may begin your investigation 30 days after the date FDA received your submission, unless FDA notifies you that your investigation may not begin.

Any administrative questions concerning this submission should be directed to the IDE staff at (301) 427-1190. Any future correspondence regarding this submission should be identified with your IDE number and should be submitted, in triplicate, to:

Food and Drug Administration
Center for Devices and
Radiological Health
Document Mail Center (HFZ-401)
1390 Piccard Drive
Rockville, Maryland 20850

Sincerely,

Deborah Falls

Nancy F. Teague, M.P.H.
Chief, IDE Section
Program Operations Staff
Office of Device Evaluation
Center for Devices and
Radiological Health

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
1390 Piccard Drive
Rockville MD 20850

NOV 14 1991

Samuel Yin, Ph.D.
Director, Biotech Division
Stryker Corporation
2725 Fairfield Road
Kalamazoo, Michigan 49002

Re: IDE Number G910130/A2
Stryker Osteogenic Protein (OP) Device
Dated: October 17, 1991
Received: October 18, 1991

Dear Dr. Yin:

The Food and Drug Administration (FDA) has reviewed the amendment to your investigational device exemptions (IDE) application. Your application is conditionally approved because you have not adequately addressed some of the deficiencies in our October 11, 1991 disapproval letter. You may begin your investigation after you have obtained institutional review board (IRB) approval and submitted certification of IRB approval to FDA. Your investigation is limited to 10 institutions and 150 patients.

This approval is being granted on the condition that, within 30 days from the date of this letter, you submit information correcting the following deficiencies:

REDACTED

REDACTED

REDACTED

If you agree to these conditions, you may begin an investigation at a new investigational site after the IRB has approved the investigation. No documentation should be submitted for any institution within the approved limit until the investigational site limit is reached or the 6-month current investigator list is due. You must submit a supplemental IDE application, and receive FDA approval, prior to expanding the investigation past the limit specified above. If you do not agree to these conditions, you must comply with the full requirements of submission of a supplemental IDE application for new investigational sites (21 CFR 812.35(b)). FDA assumes that you have agreed to the conditions of this waiver unless you specifically notify us in writing of your disagreement.

We would like to point out that FDA approval of your IDE application does not imply that this investigation will develop sufficient safety and effectiveness data to assure FDA approval of a premarket approval (PMA) application for this device. You may obtain the guideline for the preparation of a PMA application, entitled "Premarket Approval (PMA) Manual", from the Division of Small Manufacturers Assistance at their toll free number (800) 638-2041 or (301) 443-6597.

REDACTED

3. Detailed records of all data pertaining to sterilization, including method validation must be kept and presented at the time of PMA application.

4. If your sample size estimated was minimal, a 5% per year expected loss to follow-up may not be adequate.

We have enclosed the guidance document entitled "Sponsor Responsibilities for a Significant Risk Device Investigation" to help you understand the functions and duties of a sponsor. Please contact the individuals listed below if you have any questions regarding these responsibilities.

If you have any questions, please call Michael J. Blackwell, D.V.M., M.P.H., at (301) 427-1036 or the IDE Staff at (301) 427-1190.

Sincerely yours,

Robert L. Sheridan
Director
Office of Device Evaluation
Center for Devices and
Radiological Health

Enclosures

PROCEDURE FORM

Form P
Page 1
Study #91-01

Patient # 1001 Patient Initials A.W.

Date of Procedure: 02 / 14 / 92
M D Y

JSI
4/15/92

☐ Received Autograft Site of Harvest _____

☒ Received Stryker OP Device Kit # 1 + 2

ACTUAL GAP SIZE FILLED:

Size of Non-Union Gap in Widest Point 10 mm

Size of Non-Union Gap in Narrowest Point 1 mm

DESCRIPTION OF IM ROD:

Type: SLOTTED Material: Stainless Steel

Length: 35 cm Diameter: 13 mm

Manufacturer: SYNTHES

Lot #: UNK

0

☒ New Rod Inserted ☐ Previous Rod Left in Place

☒ Reamed ☐ Non-Reamed

☐ Locking ☒ Non-Locking

Location:

☐ Proximal

☐ Distal

☐ Both

Total Blood loss during procedure: 100 cc

Approximate Surgery Time (Do Not Include Prep Time): 3 hrs. 0 min.

Fibulectomy ☒ Yes

Concomitant Surgical Procedures: fibulectomy

6/16/92

Hosp Dlcdate 02/17/92

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
1001	S	-2	02/03/92
	S	0	02/15/92
	S	1	03/17/92
	S	2	04/09/92
	S	3	05/14/92
	S	6	08/13/92
	S	9	11/12/92
	S	12	02/11/93
	S	24	01/19/94
	S	36	02/13/95
	S	48	05/11/96
	S	99	01/23/97
1002	S	-4	01/07/93
	S	-4	09/28/94
	S	-2	02/28/92
	S	0	02/29/92
	S	1	03/26/92
	S	2	04/20/92
	S	3	05/21/92
	S	6	08/03/92
	S	12	03/24/93
	S	48	02/07/96
1003	S	-4	05/11/92
	S	-2	02/17/92
	S	0	02/27/92
	S	1	03/26/92
	S	3	05/26/92
	S	9	12/14/92
	S	24	11/05/93
	S	99	07/27/94
	S	99	03/25/97
1004	S	-4	06/16/93
	A	-2	02/18/92
	B	-2	02/18/92

REDACTED

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
1004	A	0	03/04/92
	B	0	03/04/92
	A	1	04/01/92
	B	1	04/01/92
	A	2	05/13/92
	B	2	05/13/92
	A	3	06/09/92
	B	3	06/09/92
	A	6	09/09/92
	B	6	09/09/92
	A	9	12/16/92
	B	9	12/16/92
	A	12	03/10/93
	B	12	03/10/93
	A	24	02/10/94
	B	24	02/10/94
1005	S	-2	08/10/92
1006	S	-2	08/24/92
1007	S	-4	05/17/93
	S	-2	05/03/93
	S	0	05/07/93
	S	2	06/23/93
	S	3	07/26/93
	S	3	08/18/93
	S	6	12/01/93
	S	9	02/10/94
	S	12	05/12/94
	S	24	05/15/95
	S	36	05/14/96
1008	S	-2	03/04/93
1009	S	-2	06/29/93
	S	0	06/30/93

REDACTED

Pat1	Pat2	Visit Code	Visit Date
1009	S	1	07/26/93
	S	2	08/23/93
	S	3	10/04/93
	S	6	01/03/94
	S	9	03/21/94
	S	12	06/06/94
	S	24	07/10/95
	S	99	03/13/97
1010	S	-4	10/06/94
	S	-4	03/19/96
	S	-2	12/03/93
	S	0	12/04/93
	S	1	01/13/94
	S	2	02/08/94
	S	3	03/03/94
	S	6	06/16/94
	S	12	12/16/94
1011	S	-4	03/31/94
	S	-2	11/15/93
	S	0	11/19/93
	S	1	12/06/93
	S	2	01/24/94
	S	3	02/28/94
	S	9	07/22/94
	S	12	10/24/94
	S	24	09/07/95
1012	S	-2	05/17/94
	S	0	05/20/94
	S	1	06/09/94
	S	6	11/10/94
	S	9	03/14/95
	S	12	07/18/95
	S	24	05/21/96

REDACTED

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
1013	S	-4	11/01/94
	S	-2	10/26/94
	S	0	10/27/94
	S	1	11/28/94
	S	2	12/29/94
	S	3	01/13/95
	S	6	04/07/95
	S	9	07/14/95
	S	12	10/05/95
	S	24	08/27/96
1014	S	-2	01/23/95
	S	0	02/13/95
	S	1	03/23/95
	S	2	04/20/95
	S	3	05/18/95
	S	6	08/10/95
	S	9	11/02/95
	S	12	01/25/96
	S	24	01/27/97
1015	S	-4	04/12/95
	S	-2	04/05/95
	S	0	04/08/95
	S	1	05/02/95
	S	2	06/07/95
	S	3	07/13/95
	S	6	10/12/95
	S	9	01/24/96
	S	12	05/01/96
	S	24	06/18/97
1016	S	-2	05/17/95
	S	0	05/24/95
	S	1	07/05/95
	S	2	08/02/95
	S	9	03/06/96

REDACTED

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
1017	S	-2	05/17/95
1018	S	-4	06/20/95
	S	-2	05/24/95
	S	0	06/08/95
	S	3	08/29/95
	S	6	11/28/95
	S	9	03/12/96
	S	12	05/01/96
1019	S	-2	08/18/95
1020	S	-4	06/17/96
	S	-4	01/09/97
	S	-2	06/05/96
	S	0	06/06/96
	S	1	07/15/96
	S	2	08/15/96
	S	3	09/12/96
	S	9	02/24/97
	S	12	06/16/97
1101	S	-2	07/13/92
	S	0	07/16/92
	S	1	08/11/92
	S	2	09/15/92
	S	3	10/13/92
	S	6	01/19/93
	S	9	03/30/93
	S	12	06/04/93
	S	99	02/20/97
1102	S	-2	09/21/92
	S	0	09/24/92
	S	1	10/22/92
	S	2	11/24/92
	S	3	12/22/92

REDACTED

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
1102	S	6	03/23/93
	S	9	06/29/93
	S	12	10/05/93
	S	24	10/11/94
	S	99	02/04/97
1103	A	-2	04/12/93
	B	-2	04/12/93
	A	0	04/14/93
	B	0	04/14/93
	A	1	05/12/93
	B	1	05/12/93
	A	2	06/08/93
	B	2	06/08/93
	A	3	07/13/93
	B	3	07/13/93
	A	6	10/12/93
	B	6	10/12/93
	A	9	02/08/94
	B	9	02/08/94
	A	12	04/12/94
	B	12	04/12/94
	A	24	04/04/95
	B	24	04/04/95
	A	99	02/06/96
	A	99	02/03/97
	B	99	02/06/96
	B	99	02/03/97
1104	S	-2	04/21/93
1105	S	-2	06/21/93
	S	0	07/01/93
	S	1	07/22/93
	S	2	08/19/93
	S	3	09/14/93
	S	6	11/30/93

REDACTED

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
1105	S	9	03/10/94
	S	12	06/09/94
1106	S	-2	04/03/94
	S	0	04/05/94
	S	1	04/21/94
	S	2	05/24/94
1201	S	-4	01/28/94
	S	-2	09/08/92
	S	0	09/25/92
	S	1	10/27/92
	S	2	11/10/92
	S	3	12/22/92
	S	6	04/20/93
	S	12	09/01/93
	S	99	11/01/95
	S	99	02/13/96
	S	99	09/13/96
1202	S	-2	12/08/92
	S	0	12/10/92
	S	1	01/05/93
	S	2	02/05/93
	S	3	03/09/93
	S	6	06/08/93
	S	9	09/14/93
	S	12	11/23/93
	S	24	01/09/95
	S	99	09/18/96
	S	99	11/19/97
1203	S	-4	09/19/94
	S	-2	01/25/94
	S	0	01/29/94
	S	1	03/01/94
	S	3	04/29/94

REDACTED

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
1203	S	9	10/31/94
	S	12	03/06/95
	S	99	11/19/97
	S	99	08/05/98
1204	S	-2	03/08/94
	S	0	03/11/94
	S	1	04/15/94
	S	2	05/17/94
	S	3	06/21/94
	S	6	09/19/94
	S	9	01/03/95
	S	12	03/27/95
	S	99	07/23/96
	S	99	09/18/96
	S	99	12/09/97
1205	S	-2	03/15/94
	S	0	03/18/94
	S	1	04/15/94
	S	2	05/17/94
	S	3	06/21/94
	S	6	09/19/94
	S	9	01/09/95
	S	12	03/13/95
	S	24	03/27/96
	S	99	07/30/97
1206	S	-2	05/03/94
1207	S	-4	09/18/95
	S	-2	01/23/95
	S	0	01/27/95
	S	1	02/20/95
	S	2	03/27/95
	S	3	05/01/95
	S	9	11/20/95

REDACTED

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
1207	S	12	02/28/96
	S	24	02/05/97
	S	99	11/19/97
1208	S	-2	10/16/95
	S	0	10/18/95
	S	1	11/22/95
	S	2	12/18/95
	S	3	01/24/96
	S	6	04/30/96
	S	9	07/24/96
	S	12	10/16/96
	S	24	10/29/97
1209	S	-4	04/03/96
	S	-2	12/18/95
	S	0	12/21/95
	S	1	01/17/96
	S	2	02/28/96
	S	6	05/29/96
	S	9	08/28/96
	S	12	12/16/96
	S	24	10/22/97
	S	99	01/22/97
	S	99	02/28/97
	S	99	05/30/97
1301	S	-4	08/13/92
	S	-4	10/04/94
	S	-2	03/17/92
	S	0	03/26/92
	S	1	04/16/92
	S	2	06/04/92
	S	9	01/19/93
	S	12	03/16/93
	S	99	06/11/96

REDACTED

Pat1	Pat2	Visit Code	Visit Date
1302	S	-4	02/26/93
	S	-2	03/26/92
	S	0	04/16/92
	S	1	06/14/92
	S	2	06/16/92
	S	3	07/14/92
	S	6	10/13/92
	S	12	04/01/93
	S	24	06/14/94
1303	S	-2	07/01/92
	S	0	07/02/92
	S	1	07/23/92
	S	2	08/20/92
	S	3	09/24/92
	S	6	01/19/93
	S	9	03/16/93
	S	12	06/22/93
	S	24	07/18/94
	S	99	06/20/95
1304	S	-2	06/18/92
	S	0	06/23/92
	S	1	07/30/92
	S	2	08/20/92
	S	3	09/24/92
	S	6	12/24/92
	S	9	03/23/93
	S	12	06/17/93
	S	24	06/30/94
	S	99	09/12/95
1305	S	-2	07/14/92
	S	0	07/17/92
	S	1	08/11/92
	S	2	09/10/92
	S	3	10/13/92

REDACTED

Pat1	Pat2	Visit Code	Visit Date
1305	S	6	02/09/93
	S	9	04/01/93
	S	12	08/05/93
	S	24	05/24/94
	S	99	09/17/96
1306	S	-2	07/21/92
	S	0	07/28/92
	S	1	08/20/92
	S	2	09/17/92
	S	3	10/15/92
	S	6	02/04/93
	S	9	04/01/93
	S	12	07/01/93
	S	24	07/12/94
	S	99	05/02/95
1307	S	-4	12/22/93
	S	-2	01/28/93
	S	0	02/02/93
	S	1	03/02/93
	S	2	04/01/93
	S	3	04/29/93
	S	6	08/31/93
	S	12	02/08/94
	S	24	03/16/95
1308	S	-4	07/13/94
	S	-2	03/02/93
	S	0	03/21/93
	S	1	04/20/93
	S	2	05/13/93
	S	3	06/10/93
	S	6	09/09/93
	S	9	12/14/93
	S	12	03/29/94
	S	24	03/14/95

REDACTED

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
1308	S	99	03/26/96
	S	99	12/16/97
1309	S	-4	05/10/93
	S	-2	05/06/93
	S	0	05/12/93
	S	1	06/17/93
	S	2	07/15/93
	S	3	08/12/93
	S	6	11/16/93
	S	9	02/08/94
	S	12	04/07/94
	S	24	05/09/95
1310	S	-4	09/20/93
	S	-2	09/16/93
	S	1	10/19/93
	S	2	11/11/93
	S	3	12/21/93
	S	6	03/01/94
	S	12	09/01/94
	S	99	12/03/96
	S	99	12/11/97
1311	S	-4	05/26/94
	S	-4	11/07/94
	S	-2	09/21/93
	S	0	09/23/93
	S	2	11/09/93
	S	3	12/21/93
	S	9	07/21/94
	S	12	09/22/94
	S	24	09/21/95
1312	S	-4	04/19/94
	S	-2	11/29/93
	S	0	11/30/93

REDACTED

Pat1	Pat2	Visit Code	Visit Date
1312	S	1	01/04/94
	S	2	02/08/94
	S	6	06/16/94
	S	9	08/16/94
	S	12	12/15/94
	S	99	12/18/97
1313	S	-4	09/27/94
	S	-4	02/29/96
	S	-2	11/29/93
	S	0	11/30/93
	S	1	01/06/94
	S	2	02/10/94
	S	3	03/03/94
	S	6	06/14/94
	S	9	08/16/94
	S	12	11/17/94
	S	99	09/12/96
	S	99	01/13/98
1314	S	-2	03/08/94
	S	0	03/13/94
	S	1	04/07/94
	S	2	05/03/94
	S	3	06/14/94
	S	6	09/13/94
	S	9	12/13/94
	S	12	03/07/95
	S	24	03/05/96
	S	99	12/09/97
1315	S	-4	08/23/94
	S	-4	01/18/95
	S	-4	01/24/95
	S	-2	07/21/94
	S	0	08/11/94
	S	2	10/04/94

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
1315	S	3	11/10/94
	S	6	03/07/95
	S	9	05/02/95
	S	12	08/31/95
	S	24	08/22/96
	S	99	12/18/97
1316	S	-2	11/03/94
	S	0	11/08/94
	S	1	11/29/94
	S	2	01/10/95
	S	3	02/09/95
	S	6	06/06/95
	S	9	07/27/95
	S	12	11/02/95
1401	S	-2	09/02/92
	S	0	09/04/92
	S	1	09/27/92
	S	2	10/26/92
	S	3	12/10/92
	S	6	03/08/93
	S	9	06/07/93
	S	12	09/24/93
	S	24	10/12/94
1402	S	-4	04/18/94
	S	-4	09/11/95
	S	-2	06/02/93
	S	0	06/04/93
	S	1	06/28/93
	S	2	07/29/93
	S	3	08/25/93
	S	6	12/08/93
	S	9	02/28/94
	S	12	07/18/94

REDACTED

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
1403	S	-2	07/15/93
	S	0	07/16/93
	S	1	08/12/93
	S	2	09/16/93
	S	3	10/19/93
	S	6	01/17/94
	S	9	04/18/94
	S	12	07/18/94
	S	24	09/18/95
1404	S	-2	01/24/94
	S	0	01/26/94
	S	1	02/21/94
	S	2	03/28/94
	S	3	05/02/94
	S	6	06/27/94
	S	9	10/03/94
	S	12	02/06/95
	S	24	01/08/96
	S	99	01/13/97
1405	S	-4	07/18/94
	S	-4	09/02/94
	S	-4	08/06/96
	S	-2	04/29/94
	S	0	05/05/94
	S	1	06/06/94
	S	9	02/06/95
	S	12	05/01/95
1406	S	-2	10/05/94
1407	S	-4	02/13/95
	S	-4	01/03/96
	S	-2	10/26/94
	S	0	10/28/94
	S	1	11/23/94

REDACTED

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
1407	S	2	12/28/94
	S	6	05/15/95
	S	9	07/24/95
1408	S	-4	09/11/95
	S	-2	10/26/94
	S	0	11/02/94
	S	1	11/30/94
	S	2	12/26/94
	S	3	02/06/95
	S	6	05/08/95
	S	12	11/06/95
1409	S	-4	02/15/96
	S	-2	04/12/95
	S	0	04/14/95
	S	1	05/15/95
	S	2	06/12/95
	S	3	07/17/95
	S	6	09/26/95
	S	9	01/22/96
	S	12	04/22/96
	S	24	04/09/97
1410	S	-4	11/11/96
	S	-2	07/25/96
	S	0	07/26/96
	S	1	08/26/96
	S	2	09/25/96
	S	6	01/22/97
	S	12	07/16/97
1501	S	-4	05/01/92
	S	-4	08/20/92
	S	-4	02/04/93
	S	-4	02/08/93
	S	-2	04/03/92

REDACTED

Pat1	Pat2	Visit Code	Visit Date
1501	S	1	05/15/92
	S	2	06/19/92
	S	3	07/21/92
	S	6	10/20/92
	S	9	12/22/92
	S	12	05/06/93
	S	24	02/17/94
	S	36	05/23/95
	S	48	03/21/96
	S	99	11/20/97
1502	S	-2	07/22/92
	S	0	07/23/92
	S	1	08/20/92
	S	2	09/22/92
	S	3	10/20/92
	S	6	01/19/93
	S	9	04/29/93
	S	12	07/27/93
	S	24	07/22/94
	S	99	08/04/95
	S	99	08/08/96
	S	99	11/21/97
1503	S	-2	08/04/93
	S	0	08/07/93
	S	1	09/07/93
	S	2	09/28/93
	S	3	10/26/93
	S	6	02/17/94
	S	9	05/05/94
	S	12	08/09/94
	S	24	08/15/95
	S	99	09/27/96
1504	S	-2	08/18/92

REDACTED

002525

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
1505	S	-2	05/06/93
	S	0	05/06/93
	S	1	06/03/93
	S	2	07/01/93
	S	3	08/06/93
	S	6	11/05/93
	S	9	02/22/94
	S	12	05/10/94
	S	24	05/19/95
	S	99	05/21/96
	S	99	11/13/97
1506	S	-2	07/15/93
	S	0	08/06/93
	S	1	09/02/93
	S	2	10/01/93
	S	3	11/02/93
	S	6	02/03/94
	S	9	05/17/94
	S	12	07/19/94
	S	24	08/15/95
	S	99	09/06/96
	S	99	01/13/98
1507	S	-2	09/01/93
	S	0	09/02/93
	S	1	10/01/93
	S	2	11/02/93
	S	3	12/07/93
	S	6	03/15/94
	S	9	05/26/94
	S	12	10/06/94
	S	24	08/24/95
	S	99	08/27/96
	S	99	12/04/97
1508	S	-4	04/19/94

REDACTED

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
1508	S	-4	06/23/94
	S	-4	07/25/94
	S	-4	09/06/94
	S	-2	04/08/94
	S	1	05/10/94
	S	6	10/07/94
	S	9	01/06/95
	S	12	05/02/95
	S	24	04/05/96
1509	S	-2	05/05/94
	S	0	05/06/94
	S	1	06/03/94
	S	2	07/08/94
	S	3	08/04/94
	S	6	11/10/94
	S	9	02/10/95
	S	12	05/19/95
	S	24	06/04/96
	S	99	11/25/97
1510	S	-2	08/25/94
	S	0	09/15/94
	S	1	10/25/94
	S	2	11/15/94
	S	3	12/13/94
	S	6	03/07/95
	S	24	09/20/96
1511	S	-2	10/17/95
1601	S	-4	03/03/93
	S	-4	07/23/93
	S	-4	10/11/94
	S	-2	02/23/93
	S	0	02/27/93
	S	1	03/22/93

REDACTED

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
1601	S	2	04/22/93
	S	3	06/02/93
	S	6	09/14/93
	S	9	11/19/93
	S	12	01/17/94
	S	24	02/10/95
	S	99	12/04/95
	S	99	06/16/97
1602	S	-2	11/27/95
1701	S	-2	10/05/92
	S	0	10/06/92
	S	1	11/10/92
	S	2	12/10/92
	S	3	01/12/93
	S	6	04/09/93
	S	9	07/16/93
	S	12	09/01/93
	S	24	12/02/94
	S	99	01/03/96
1702	S	-4	01/29/93
	S	-2	12/08/92
	S	0	12/11/92
	S	1	01/05/93
	S	2	02/11/93
	S	3	03/11/93
	S	6	06/15/93
	S	9	10/06/93
	S	12	12/13/93
	S	24	12/13/94
	S	99	03/07/96
1703	S	-2	03/30/94
	S	0	04/02/94
	S	1	04/26/94

REDACTED

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
1703	S	2	05/25/94
	S	3	07/12/94
	S	6	09/20/94
	S	9	12/27/94
	S	12	03/31/95
	S	24	04/11/96
1704	S	-4	11/12/93
	S	-2	11/08/93
	S	1	12/08/93
	S	2	01/11/94
	S	3	02/18/94
	S	6	05/24/94
	S	9	08/16/94
	S	12	12/05/94
	S	99	04/26/96
1705	S	-4	12/03/93
	S	-2	11/16/93
	S	0	11/19/93
	S	2	01/04/94
	S	3	02/15/94
	S	6	05/17/94
	S	9	08/16/94
	S	12	11/08/94
	S	24	11/17/95
1706	S	-4	05/25/94
	S	-2	02/08/94
	S	0	02/11/94
	S	1	03/22/94
	S	2	04/13/94
	S	6	08/15/94
	S	9	11/15/94
	S	12	02/02/95
1707	S	-4	06/06/94

REDACTED

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
1707	S	-2	04/19/94
	S	0	04/24/94
	S	1	05/12/94
	S	3	07/12/94
	S	6	10/27/94
	S	9	01/11/95
	S	12	05/16/95
	S	24	06/04/96
1708	S	-2	07/15/94
	S	0	07/19/94
	S	1	08/04/94
	S	2	09/20/94
	S	3	10/18/94
	S	6	01/12/95
	S	9	04/18/95
	S	12	08/18/95
1709	S	-2	04/11/95
	S	0	04/28/95
	S	1	05/26/95
	S	2	07/11/95
	S	3	08/01/95
	S	6	11/17/95
	S	9	02/13/96
	S	12	04/30/96
1710	S	-2	03/23/96
	S	0	03/27/96
	S	1	04/22/96
	S	2	05/22/96
	S	3	07/08/96
	S	6	10/16/96
	S	9	01/13/97
1801	S	-2	09/28/92

REDACTED

Pat1	Pat2	Visit Code	Visit Date
1901	S	-2	06/16/93
	S	0	06/17/93
	S	1	07/08/93
	S	2	08/09/93
	S	3	09/09/93
	S	6	12/28/93
	S	9	03/30/94
	S	12	07/18/94
	S	24	06/08/95
	S	99	03/06/97
1902	S	-4	10/19/94
	S	-2	06/24/93
	S	0	07/08/93
	S	1	08/05/93
	S	2	09/09/93
	S	3	10/07/93
	S	6	01/27/94
	S	9	04/21/94
	S	12	07/14/94
	S	24	07/06/95
	S	99	12/10/97
1903	S	-4	12/02/93
	S	-2	07/08/93
	S	0	07/30/93
	S	1	08/19/93
	S	6	01/12/94
	S	9	04/07/94
1904	S	-2	07/20/93
	S	0	07/23/93
	S	1	08/19/93
	S	2	09/16/93
	S	3	10/21/93
	S	6	01/13/94
	S	9	05/12/94

REDACTED

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
1904	S	12	08/16/94
	S	24	07/20/95
	S	99	12/05/97
1905	S	-4	08/18/93
	S	-2	08/12/93
	S	0	08/14/93
	S	1	09/22/93
	S	2	10/18/93
	S	3	11/11/93
	S	6	03/03/94
	S	9	05/05/94
	S	12	08/11/94
	S	24	08/03/95
	S	99	08/13/96
	S	99	12/14/97
1906	S	-4	04/04/94
	S	-4	07/20/94
	S	-2	03/23/94
	S	2	05/19/94
	S	3	06/23/94
	S	6	09/29/94
	S	9	01/26/95
	S	12	04/27/95
	S	24	02/22/96
1907	S	-2	05/19/94
	S	0	05/25/94
	S	1	06/29/94
	S	2	07/14/94
	S	3	08/18/94
	S	6	12/05/94
	S	9	02/23/95
	S	12	06/29/95
1908	S	-4	04/13/95

REDACTED

Pat1	Pat2	Visit Code	Visit Date
1908	S	-2	09/07/94
	S	0	09/09/94
	S	1	09/29/94
	S	2	11/10/94
	S	3	12/08/94
	S	9	06/08/95
	S	12	08/24/95
2001	S	-4	02/24/93
	S	-4	08/17/94
	S	-4	11/16/94
	S	-2	02/09/93
	S	0	02/13/93
	S	2	04/07/93
	S	3	05/05/93
	S	6	08/25/93
	S	9	12/08/93
	S	12	03/09/94
	S	24	01/11/95
	S	99	05/01/96
	S	99	11/03/97
2002	S	-4	03/17/93
	S	-4	05/19/93
	S	-4	06/22/95
	S	-2	02/24/93
	S	0	03/05/93
	S	2	04/21/93
	S	3	06/18/93
	S	6	09/15/93
	S	9	11/17/93
	S	12	02/16/94
	S	99	12/05/97
2003	S	-4	05/26/93
	S	-4	09/01/93
	S	-4	12/01/93

REDACTED

Pat1	Pat2	Visit Code	Visit Date
2003	S	-4	02/01/94
	S	-4	05/26/94
	S	-2	03/31/93
	S	0	04/13/93
	S	1	04/28/93
	S	2	06/16/93
	S	3	06/30/93
	S	9	01/26/94
	S	12	03/30/94
	S	24	05/10/95
	S	99	12/03/97
2004	S	-2	01/18/94
	S	0	02/08/94
	S	1	02/23/94
	S	2	03/30/94
	S	3	05/17/94
	S	6	08/10/94
	S	12	01/12/95
	S	24	01/10/96
	S	99	01/25/96
	S	99	12/05/97
2005	S	-2	06/16/93
	S	0	06/29/93
	S	1	07/14/93
	S	1	08/11/93
	S	2	09/08/93
	S	6	12/08/93
	S	9	03/09/94
	S	12	06/06/94
	S	24	06/14/95
	S	99	12/16/97
2006	S	-2	09/01/93
2007	S	-4	10/27/93

REDACTED

Pat1	Pat2	Visit Code	Visit Date
2007	S	-4	05/18/94
	S	-4	02/08/95
	S	-2	09/22/93
	S	0	10/12/93
	S	1	11/24/93
	S	3	01/05/94
	S	6	03/16/94
	S	12	09/28/94
	S	24	01/03/96
	S	99	02/29/96
	S	99	12/30/96
	S	99	12/16/97
2008	S	-4	11/03/93
	S	-2	09/29/93
	S	0	10/19/93
	S	2	12/08/93
	S	3	01/12/94
	S	6	04/20/94
	S	9	07/13/94
	S	12	10/05/94
	S	24	11/22/95
	S	99	12/30/96
	S	99	12/04/97
2009	S	-4	11/30/93
	S	-4	06/22/94
	S	-2	11/10/93
	S	0	11/16/93
	S	2	01/12/94
	S	3	02/16/94
	S	9	08/31/94
	S	12	11/16/94
2010	S	-4	12/22/93
	S	-2	11/30/93
	S	0	12/07/93

REDACTED

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
2010	S	2	01/26/94
	S	3	03/09/94
	S	6	06/22/94
	S	9	09/21/94
	S	12	12/07/94
	S	24	12/06/95
	S	99	12/30/96
	S	99	12/15/97
2011	S	-2	01/25/94
	S	0	02/16/94
	S	1	03/02/94
	S	2	04/13/94
	S	3	05/18/94
	S	6	08/31/94
	S	9	11/30/94
	S	12	02/15/95
	S	24	04/03/96
2012	S	-4	03/30/94
	S	-2	03/10/94
	S	0	03/18/94
	S	2	05/11/94
	S	3	06/22/94
	S	6	08/31/94
	S	9	11/30/94
	S	12	03/01/95
	S	24	03/21/96
	S	99	12/08/97
2013	S	-4	07/27/94
	S	-4	02/23/95
	S	-2	06/22/94
	S	0	07/12/94
	S	1	08/24/94
	S	2	09/21/94
	S	9	04/19/95

REDACTED

Pat1	Pat2	Visit Code	Visit Date
2013	S	12	08/16/95
	S	24	07/17/96
	S	99	12/22/97
2014	S	-4	08/10/94
	S	-4	01/25/95
	S	-4	02/22/95
	S	-4	08/30/95
	S	-2	07/12/94
	S	0	07/26/94
	S	2	09/21/94
	S	3	10/26/94
	S	6	12/28/94
	S	9	03/29/95
	S	12	06/28/95
	S	24	07/10/96
	S	99	11/26/97
2015	S	-4	08/03/94
	S	-2	07/12/94
	S	0	07/19/94
	S	1	08/31/94
	S	2	09/28/94
	S	6	12/28/94
	S	9	03/29/95
	S	12	06/28/95
	S	24	07/31/96
	S	99	12/10/97
2016	S	-4	10/13/94
	S	-2	08/30/94
	S	0	09/13/94
	S	1	09/28/94
2017	S	-4	11/09/94
	S	-2	09/06/94
	S	0	09/27/94

REDACTED

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
2017	S	1	10/12/94
	S	3	12/14/94
	S	6	03/30/95
	S	9	06/21/95
	S	12	10/18/95
	S	24	09/25/96
2018	S	-4	03/29/95
	S	-4	07/12/95
	S	-2	02/01/95
	S	0	02/14/95
	S	1	03/01/95
	S	2	04/12/95
	S	3	05/17/95
	S	6	08/16/95
	S	12	02/28/96
	S	24	05/24/96
2019	S	-4	10/04/95
	S	-4	04/10/96
	S	-2	05/17/95
	S	0	06/06/95
	S	1	06/21/95
	S	2	08/16/95
	S	6	11/29/95
	S	12	06/26/96
2101	S	-2	08/24/93
	S	0	09/09/93
	S	1	09/28/93
	S	2	11/02/93
	S	3	11/30/93
	S	6	02/22/94
	S	9	05/17/94
	S	12	08/23/94
	S	24	09/14/95
	S	99	03/28/97

REDACTED

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
2201	S	-4	11/09/94
	S	-2	07/06/94
	S	0	07/08/94
	S	3	09/26/94
	S	6	01/10/95
2202	S	-4	08/26/96
	S	-2	10/11/94
	S	0	10/12/94
	S	1	11/03/94
	S	2	12/01/94
	S	3	01/19/95
	S	6	04/27/95
	S	9	07/27/95
	S	12	10/12/95
	S	24	07/25/96
2203	S	-4	04/22/96
	S	-4	02/19/97
	S	-2	11/30/95
	S	0	12/13/95
	S	1	01/23/96
	S	2	02/07/96
	S	3	03/13/96
	S	9	09/09/96
2301	S	-2	01/17/94
	S	0	01/18/94
	S	1	02/25/94
	S	2	03/23/94
	S	3	04/20/94
	S	6	08/03/94
	S	9	11/16/94
	S	12	01/24/95
	S	24	01/30/96
	S	99	10/24/96

REDACTED

Pat1	Pat2	Visit Code	Visit Date
2601	S	-4	03/02/94
	S	-2	12/10/93
	S	0	12/16/93
	S	1	01/05/94
	S	2	02/11/94
	S	3	03/25/94
	S	6	06/01/94
	S	9	10/07/94
	S	12	01/04/95
	S	24	10/04/95
	S	99	12/10/96
2602	S	-4	05/18/94
	S	-2	04/26/94
	S	0	05/04/94
	S	1	06/14/94
	S	3	08/01/94
	S	6	11/16/94
	S	9	02/15/95
	S	12	03/15/95
2901	S	-2	04/13/94
	S	0	04/18/94
	S	1	05/11/94
	S	2	06/08/94
	S	3	07/13/94
	S	6	10/12/94
	S	9	01/11/95
	S	12	04/12/95
	S	24	04/10/96
	S	36	03/31/97
	S	99	12/18/97
2902	S	-2	07/08/98
2903	S	-4	09/04/95
	S	-2	01/04/95

REDACTED

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
2903	S	0	01/11/95
	S	1	02/17/95
	S	2	03/17/95
	S	3	04/17/95
	S	6	07/10/95
	S	12	02/15/96
	S	24	01/12/97
2904	S	-4	08/07/96
	S	-2	01/25/95
	S	0	02/06/95
	S	1	03/08/95
	S	2	03/29/95
	S	6	08/04/95
	S	12	01/18/96
2905	S	-2	09/07/95
	S	0	09/14/95
	S	1	10/16/95
	S	6	04/08/96
3001	S	-2	03/03/94
	S	0	03/10/94
	S	1	04/06/94
	S	2	05/04/94
	S	3	06/01/94
	S	6	09/14/94
	S	9	12/21/94
	S	12	03/09/95
	S	24	04/03/96
	S	36	11/21/97
3002	S	-4	06/13/96
	S	-2	10/19/94
	S	0	10/29/94
	S	1	11/30/94
	S	2	12/21/94

REDACTED

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
3002	S	3	01/19/95
	S	6	04/26/95
	S	9	08/02/95
	S	12	10/25/95
	S	24	10/30/96
	S	99	11/24/97
3003	S	-4	02/02/96
	S	-2	05/03/95
	S	0	05/21/95
	S	1	06/14/95
	S	2	07/13/95
	S	3	08/05/95
	S	6	11/16/95
	S	9	01/22/96
	S	12	05/09/96
	S	24	06/03/97
3004	S	-2	08/23/95
	S	0	08/31/95
	S	1	09/20/95
	S	3	11/14/95
	S	6	03/07/96
	S	9	05/02/96
3005	S	-2	08/05/96
	S	0	08/09/96
	S	1	09/10/96
	S	2	10/15/96
	S	3	11/12/96
	S	6	02/17/97
	S	9	05/30/97
	S	12	09/19/97
	S	24	08/17/98
3101	S	-2	09/16/94
	S	0	09/30/94

REDACTED

Pat1	Pat2	Visit Code	Visit Date
3101	S	1	10/25/94
	S	2	11/29/94
	S	3	01/10/95
	S	6	03/31/95
	S	9	06/30/95
	S	12	11/14/95
	S	24	12/23/96
	S	99	12/12/97
3102	S	-2	10/12/94
	S	0	10/13/94
	S	1	11/22/94
	S	2	12/04/94
	S	3	01/10/95
	S	6	04/11/95
	S	9	07/18/95
	S	12	10/17/95
	S	24	10/18/96
	S	36	12/05/97
3103	S	-2	03/21/95
	S	0	04/11/95
	S	1	05/02/95
	S	2	06/06/95
	S	3	07/11/95
	S	6	10/13/95
	S	9	01/19/96
	S	12	04/12/96
	S	24	04/18/97
	S	99	12/05/97
3104	S	-2	07/11/95
3105	S	-2	11/07/95
	S	0	11/21/95
	S	1	12/12/95
	S	2	01/16/96

REDACTED

Listing: CLINPHYS Dataset

Pat1	Pat2	Visit Code	Visit Date
3105	S	3	03/05/96
	S	6	05/14/96
	S	9	08/12/96
	S	12	11/12/96
	S	24	12/12/97
3106	S	-4	06/20/97
	S	-4	10/10/97
	S	-2	01/09/96
	S	0	01/31/96
	S	1	02/20/96
	S	2	03/15/96
	S	3	03/29/96
	S	6	07/30/96
	S	9	10/29/96
	S	12	01/26/97
	S	24	01/09/98
	S	99	11/21/97
3107	S	-2	02/16/96
	S	0	02/26/96
	S	1	03/22/96
	S	2	04/23/96
	S	3	05/28/96
	S	6	08/27/96
	S	9	11/26/96
	S	12	03/04/97
	S	24	04/13/98
	S	99	12/05/97
3108	S	-2	08/09/96
	S	0	08/13/96
	S	1	09/10/96
	S	2	10/15/96
	S	3	11/22/96
	S	6	03/10/97
	S	9	06/12/97

REDACTED

Pat1	Pat2	Visit Code	Visit Date
3108	S	12	08/29/97
	S	99	12/05/97

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DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
9200 Corporate Boulevard
Rockville MD 20850

Amy J. LaForte, Ph.D.
Regulatory Affairs Manager
Stryker Biotech
190 N. Main St.
Natick, MA 01760

MAY 19 1998

Dear Dr. LaForte:

The Center for Devices and Radiological Health (CDRH) of the Food and Drug Administration (FDA) acknowledges receipt of your Premarket Approval Modular Submission. This modular submission has been assigned the following unique document control number. Failure to reference this assigned number in future correspondence may result in processing delays.

Modular PMA Submission : M980003
Dated: April 4, 1998
Received: April 4, 1998
Device: NOVOS™

The PMA Modular Program is a new CDRH initiative and guidance is currently being drafted. This draft guidance will become available shortly and will be placed on the Internet on FDA's HomePage. Any additional information should be clearly identified as for modular review and should cite the above tracking number. Please contact the Premarket Approval Section of the Program Operations Staff at (301) 594-2186, if you have any questions.

Future correspondence regarding this PMA modular submission should be submitted with the required number of copies to:

Document Mail Center (HFZ-401)
Center for Devices and Radiological Health
Food and Drug Administration
9200 Corporate Blvd.
Rockville, Maryland 20850

Sincerely yours,

Kathy Poneleit
Director, Premarket Approval Section
Program Operations Staff
Office of Device Evaluation
Center for Devices and
Radiological Health

stryker®
BIOTECH

May 21, 1998

Food and Drug Administration
Document Mail Center (HFZ-401)
9200 Corporate Boulevard
Rockville, MD 20850

Re: NOVOS™ Pre-PMA, Device Characteristics and Manufacturing Module

Dear Sir/Madam:

Enclosed are three copies of the Device Characteristics and Manufacturing Module. This module is the second module describing NOVOS. The first module was

M980003, Nonclinical Laboratory Studies Pre-PMA Module

and is currently under review. Both of these modules reference IDE G910130 and Supplements. Specifically, this manufacturing module references M980003 and the following:

G910130, Original IDE and Supplements 5, 7, 20, 29, 30 and 37.

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190 North Main Street Natick, MA 01760 Voice: +1-508-653-2280 Fax: +1-508-653-2770

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Stryker Biotech considers all information provided in this document to be CONFIDENTIAL and PROPRIETARY and not to be disclosed under Freedom of Information.

Please feel free to call me at (508) 653-2280 if you have any questions.

Sincerely,
Stryker Biotech

Amy J. LaForte, Ph.D.
Regulatory Affairs Manager

OP-1

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
9200 Corporate Boulevard
Rockville MD 20850

Amy J. LaForte, Ph.D.
Regulatory Affairs Manager
Stryker Biotech
190 North Main Street
Natick, Massachusetts 01760

AUG 21 1998

Re: M980003/M001
NOVOS™ Nonclinical Laboratory Studies Module
Received: April 8, 1998
Amended: April 24, June 16 and 17, and July 31, 1998

Dear Ms. LaForte:

The Center for Devices and Radiological Health (CDRH) of the Food and Drug Administration (FDA) has completed its evaluation of your premarket approval application (PMA) modular submission, containing the report of the Nonclinical Laboratory Studies for your device. The module is accepted, and is now considered closed.

If a change is made to your device that requires the submission of additional information to this module, prior to the submission of your PMA application, you should submit the information in the form of a supplement to this module. We encourage you to consult with the Office of Device Evaluation before supplementing a closed module. Otherwise, your future PMA application should incorporate by reference the information contained in the module and should clearly identify any additional changes to the device that have not been identified in a supplement, but may affect the content of the module. You are advised that acceptance of this module does not guarantee that the application will ultimately be approved.

For a module supplement, please submit 4 copies to the address below, referencing the above PMA Shell and Module numbers:

Document Mail Center (HFZ-401)
Center for Devices and Radiological Health
Food and Drug Administration
9200 Corporate Boulevard
Rockville, MD 20850

We acknowledge your submission of draft labeling for your device. Although we have reviewed the draft labeling, we believe that it is too premature to discuss the contents since this will depend on the clinical data presented in a future PMA submission.

If you have any questions concerning this letter, please contact Angel S. Torres-Cabassa, Ph.D., at (301) 594-1296.

Sincerely yours,

Kimber Richter for

Celia M. Witten, Ph.D., M.D.
Director
Division of General and
Restorative Devices
Office of Device Evaluation
Center for Devices and
Radiological Health

DEPARTMENT OF HEALTH AND HUMAN SERVICES Public Health Service

Food and Drug Administration
Center for Devices and
Radiological Health
Office of Device Evaluation
Document Mail Center (HFZ-401)
9200 Corporate Blvd.
Rockville, Maryland 20850

June 07, 1999

AMY J. LA FORTE, PH.D.
STRYKER BIOTECH
35 SOUTH STREET
HOPKINTON, MA 01748

Dear MS. LA FORTE, PH.D.:

The Center for Devices and Radiological Health (CDRH) of the Food and Drug Administration (FDA) acknowledges receipt of your PMA ORIGINAL. This PMA ORIGINAL has been assigned the following unique document control number. Failure to reference this assigned number in future correspondence may result in processing delays.

PMA Number: P990029
Dated: 04-JUN-1999
Received: 07-JUN-1999
Device: OP-1 IMPLANT (NOVOS)

Any questions concerning this submission should be directed to the undersigned at (301)594-1184. All future correspondence regarding this PMA should be identified with the PMA number assigned above and should be submitted with the required number of copies to:

PMA Document Mail Center (HFZ-401)
Center for Devices and Radiological Health
Food and Drug Administration
9200 Corporate Blvd.
Rockville, Maryland 20850

Sincerely yours,

Contractor (F)

Michael Eudy
Consumer Safety Officer
Division of General and Restorative Devices
Office of Device Evaluation
Center for Devices and Radiological Health

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
9200 Corporate Boulevard
Rockville MD 20850

JAN 29 2001

Amy LaForte, Ph.D.
Director, Regulatory Affairs
Stryker Biotech, Inc.
35 South Street
Hopkinton, Massachusetts 01748

Re: P990029
OP-1 Implant Device
Filed: June 7, 1999
Amended: June 9, 11, and 17, August 16, and September 3, 16, and 22 (2 amendments) 1999, November 1, and December 3, 1999, March 13, April 7, May 30, June 1, 14, and 16 (3 amendments), August 8 and 16, September 6 and 12, October 6, and November 22, 2000.

Dear Dr. LaForte:

The Center for Devices and Radiological Health (CDRH) of the Food and Drug Administration (FDA) has completed its review of your premarket approval application (PMA). CDRH has determined that the PMA is not approvable. The major reasons for this decision are the following:

REDACTED

REDACTED

As provided by 21 CFR 814.44(f) you may amend your PMA as requested above, withdraw the PMA, or consider this letter to be a denial of approval of the PMA under 21 CFR 814.45 and request administrative review. Any request for administrative review, either through a hearing or review by an independent advisory committee, under section 515(d)(3) and 515(g) of the Federal Food, Drug, and Cosmetic Act, must be submitted in the form of a petition for reconsideration under 21 CFR 10.33 and in accordance with the general administrative procedures under 21 CFR 10.20. Any petition for reconsideration must be submitted to the Food and Drug Administration, Dockets Management Branch (HFA-305), Room 1061, 5630 Fishers Lane, Rockville, Maryland 20852, within 30 days of your receipt of this letter. After reviewing the petition, FDA will decide whether to grant or deny the petition and will publish a notice of its decision in the FEDERAL REGISTER. If FDA grants the petition, the notice will state the issues to be reviewed, the form of the review to be used, the persons that may participate in the review, the time and place where the review will occur, and other details.

As provided under 21 CFR 814.44(g), FDA will consider this PMA to have been voluntarily withdrawn if you fail to respond in writing within 180 days of the date of this request for a PMA amendment. You may however, amend the PMA within the 180-day period to request an extension of time to respond. Any such request is subject to FDA approval and should justify the need for the extension and provide a reasonable estimate of when the requested information will be submitted. If you do not amend the PMA within the 180-day period to (1) correct the above deficiencies, or (2) request an extension of time to respond and have the request approved, any amendment submitted after the 180-day period will be considered a resubmission of the PMA and will be assigned a new number. Under these circumstances, any resubmission will be given a new PMA number and will be subject to the requirements of21 CFR 814.20.

You may amend the PMA to provide the above requested information (6 copies), voluntarily withdraw the PMA (3 copies), direct CDRH to complete processing the PMA without the submission of additional information, or request an extension.

The required copies of the amended PMA should include the FDA reference number to facilitate processing for this PMA and should be submitted to the following address:

PMA Document Mail Center (I IFZ-401)
Center for Devices and Radiological Health
Food and Drug Administration
9200 Corporate Blvd
Rockville, Maryland 20850

If you have any questions concerning this not approvable letter, please contact Diane A. Mitchell, M.D., Acting Chief, REDB, at (301) 594-1296.

Sincerely yours,

Kimber C. Richter

Kimber C. Richter, M.D.
Deputy Director for Clinical and
Review Policy
Office of Device Evaluation
Center for Devices and
Radiological Health

stryker BIOTECH

35 South Street
Hopkinton, MA 01748
Phone (508) 416-5200
Fax (508) 416-5395

February 20, 2001

Dr. Debra Lewis
Office of Orphan Products Development (HF-35)
Food and Drug Administration
5600 Fishers Lane
Rockville, MD 20857

RE: **OP-1 Implant**
Request for Humanitarian Use Device Designation

Dear Dr. Lewis:

Pursuant to 21 CFR 814.102 (a), Stryker Biotech is requesting designation as a humanitarian use device for OP-1 Implant for the treatment of tibial nonunions in patients where previous treatment with autograft has failed or use of autograft is unfeasible. We believe that there are less than 4000 patients per year in the United States who suffer from this condition. Enclosed is documentation to support the criteria required for designation of OP-1 Implant as a humanitarian use device.

REDACTED

Please feel free to call Ms. Naseem Kabir at (508) 416-5287 or me at (508) 416-5209 if you have any questions or require further information. I look forward to hearing from you.

Sincerely,

Amy J. LaForte, Ph.D.
Director, Regulatory Affairs
Stryker Biotech

stryker
BIOTECH

35 South Street
Hopkinton, MA 01748
Phone (508) 416-5200
Fax (508) 416-5395

February 20, 2001

Dr. Debra Lewis
Office of Orphan Products Development (HF-35)
Food and Drug Administration
5600 Fishers Lane
Rockville, MD 20857

RE: OP-1 Implant
Request for Humanitarian Use Device Designation

Dear Dr. Lewis:

Pursuant to 21 CFR 814.102 (a), Stryker Biotech is requesting designation as a humanitarian use device for OP-1 Implant for the treatment of tibial nonunions in patients where previous treatment with autograft has failed or use of autograft is unfeasible. We believe that there are less than 4000 patients per year in the United States who suffer from this condition. Enclosed is documentation to support the criteria required for designation of OP-1 Implant as a humanitarian use device.

REDACTED

Please feel free to call Ms. Naseem Kabir at (508) 416-5287 or me at (508) 416-5209 if you have any questions or require further information. I look forward to hearing from you.

Sincerely,

Amy J. LaForte, Ph.D.
Director, Regulatory Affairs
Stryker Biotech

stryker
BIOTECH

35 South Street
Hopkinton, MA 01748
Phone (508) 416-5200
Fax (508) 416-5395

May 25, 2001

Food and Drug Administration/CDRH
Document Mail Center (HFZ-401)
9200 Corporate Boulevard
Rockville, MD 20850

Attention: Dr. Diane Mitchell and Jan Callaway

Re: Original HDE Submission for OP-1 Implant

Dear Dr. Mitchell and Ms. Callaway:

Please find the enclosed original application for a Humanitarian Device Exemption (HDE) for OP-1 Implant. We are including in this application a copy of the HUD designation letter from the Office of Orphan Product Development as well as unique information relevant to the HDE. As previously discussed, our PMA P990029 and amendments have been referenced by date of submission in the sections regarding technical information and the Checklist for HDE's. We are also updating this technical information with new reports pertaining to clinical, preclinical and manufacturing. These updates are provided in this HDE submission.

Please note that we have responded to the Office of Compliance regarding the remaining outstanding issues from the PMA, as we understand these issues are also relevant to the HDE. If you require a copy of the OC response, please call me with the number of copies you require and we will provide them promptly.

Enclosed are one original and 4 copies of the HDE submission. An additional copy has been sent directly to Ms. Callaway. As discussed, we will provide 4 copies of PMA P990029 in its entirety in approximately 2 weeks.

The information in both the HDE and the PMA submissions are CONFIDENTIAL and PROPRIETARY and are not to be disclosed under the Freedom of Information Act.

If you require any further information, do not hesitate to contact me at (508) 416-5209.

Sincerely,
Stryker Biotech

Amy J. LaForte, Ph.D.
Director, Regulatory Affairs

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
9200 Corporate Boulevard
Rockville MD 20850

OCT 17 2001

Amy J. LaForte, Ph.D.
Director, Regulatory Affairs
Stryker Biotech
35 South Street
Hopkinton, MA 01748

Re: H010002
OP-1™ Implant
Filed: May 29, 2001
Amended: May 29, June 28, July 5 and 23, and October 9, 11, and 17, 2001

Dear Dr. LaForte:

The Center for Devices and Radiological Health (CDRH) of the Food and Drug Administration (FDA) has completed its review of your humanitarian device exemption (HDE) application for the OP-1™ Implant. This device is indicated for use as an alternative to autograft in recalcitrant long bone nonunions where use of autograft is unfeasible and alternative treatments have failed. CDRH is pleased to inform you that your HDE is approved subject to the enclosed "Conditions of Approval." You may begin commercial distribution of the device after you have submitted an amendment to this HDE with copies of the approved labeling in final printed form.

In addition to the postapproval requirements in the enclosure, you have agreed to provide:

1. a preclinical plan for assessing the effects of OP-1™ on tumor promotion;
2. a plan for addressing the preclinical and clinical immunological commitments that you have made; and
3. a plan to collect pregnancy outcomes that will be reported in your annual report.

Please submit the study plans for the first two items within 45 days of receipt of this letter. You may submit your response to the third item within 3-6 months of receipt of this letter. The results of these postapproval studies may require modifications to be made in the labeling (via a supplement) when the studies are completed.

The sale, distribution, and use of this device are limited to prescription use in accordance with 21 CFR 801.109 within the meaning of section 520(e) of the Federal Food, Drug, and Cosmetic Act (the act) under the authority of section 515(d)(1)(B)(ii) of the act. In addition, in order to ensure the safe use of the device, FDA has further restricted the device within the meaning of section 520(e) of the act under the authority of section 515(d)(1)(B)(ii) of the act insofar as the sale, distribution, and use must not violate sections 502(q) and (r) of the act.

FDA wishes to remind you that failure to comply with the conditions of approval invalidates this approval order. Commercial distribution of a device that is not in compliance with these conditions is a violation of the act.

CDRH will notify the public of its decision to approve your HDE by making available a summary of the safety and probable benefit of the device upon which the approval was based. The information can be found on the FDA CDRH Internet HomePage located at http://www.fda.gov/cdrh/ode/hdeinfo.html. Written requests for this information can also be made to the Dockets Management Branch (HFA-305), Food and Drug Administration, 5630 Fishers Lane, Rm. 1061, Rockville, MD 20852. The written request should include the HDE number or docket number. Within 30 days from the date that this information is placed on the Internet, any interested person may seek review of this decision by requesting an opportunity for administrative review, either through a hearing or review by an independent advisory committee, under section 515(g) of the act.

You are reminded that, as soon as possible and before commercial distribution of your device, you must submit an amendment to this HDE submission with copies of all approved labeling in final printed form. As part of our reengineering effort, the Office of Device Evaluation is piloting a new process for review of final printed labeling. The labeling will not routinely be reviewed by FDA staff when HDE applicants include with their submission of the final printed labeling a cover letter stating that the final printed labeling is identical to the labeling approved in draft form. If the final printed labeling is not identical, any changes from the final draft labeling should be highlighted and explained in the amendment. Please see the CDRH Pilot for Review of Final Printed Labeling document at http://www.fda.gov/cdrh/pmat/pilotpmat.html for further details.
Any information to be submitted to FDA regarding this HDE should be submitted in triplicate, unless otherwise specified, to the address below and should reference the above HDE number to facilitate processing:

Document Mail Center (HFZ-401)
Office of Device Evaluation
Center for Devices and Radiological Health
Food and Drug Administration
9200 Corporate Blvd.
Rockville, Maryland 20850

If you have any questions concerning this approval order, please contact Ms. Jan C. Callaway at (301) 594-2018.

Sincerely yours,

Daniel G. Schultz, M.D.
Deputy Director for Clinical and Review Policy
Office of Device Evaluation
Center for Devices and Radiological Health

Enclosure
"Conditions of Approval"